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(54) **Detecting Eukaryotic Microorganisms.**

(57) A cDNA probe, for use in detecting in a sample, under conditions of predetermined stringency, a target organism belonging to a strain of fungal microorganisms, and not detecting in the sample under such conditions a reference microorganism or any prokaryotic microorganism, can be constructed by: determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from the target organism, the variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and having no corresponding region in prokaryotes; comparing the nucleotide base region in the variable region from the target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from the reference microorganism, and selecting as a useful probe site a subsequence within the determined sequence; and synthesizing a cDNA complementary to the useful probe site, the cDNA being the probe.

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
P,X	WO-A-8 803 957 (GEN-PROBE INC.) * Whole document, especially claims 1-34,241-256; page 13, lines 27-16 *	1-3	C 12 Q 1/68 C 07 H 21/04
A	-----	6,13,14	
Y	J. GEN MICROBIOL., vol. 133, no. 7, 1987, pages 1967-1974, SGM; U.B. GÖBEL et al.: "Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between Mycoplasma species" * Whole article *	1-3	
P,Y	EP-A-0 314 294 (GENE TRAK SYSTEMS) * Column 4, under heading probe development strategy *	1-3	
A	EP-A-0 232 085 (INTEGRATED GENETICS INC.) * Whole document, especially page 10, line 28 - page 11 *	1-3	
D,A	WO-A-8 402 721 (GEN-PROBE PARTNERS) * Whole document * & CA-A-1 215 904 -----	1-3,6,13, 14	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C 12 Q
The present search report has been drawn up for all claims			
Place of search		Date of completion of search	Examiner
The Hague		05 July 91	OSBORNE H.H.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document			

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54 **Detecting Eukaryotic Microorganisms.**

57 A cDNA probe, for use in detecting in a sample, under conditions of predetermined stringency, a target organism belonging to a strain of fungal microorganisms, and not detecting in the sample under such conditions a reference microorganism or any prokaryotic microorganism, can be constructed by:
determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from the target organism, the variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and having no corresponding region in prokaryotes; comparing the nucleotide base region in the variable region from the target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from the reference microorganism, and selecting as a useful probe site a subsequence within the determined sequence; and synthesizing a cDNA complementary to the useful probe site, the cDNA being the probe.

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Description

DETECTING EUKARYOTIC MICROORGANISMS

This invention relates to detecting eukaryotic microorganisms.

Fungal infection, and particularly invasive systemic candidiasis, is a significant cause of morbidity and mortality in immunocompromised patients. It is important in treatment of invasive candidiasis to obtain a diagnosis early enough to permit application of effective antifungal therapy. Known approaches to premortem diagnosis of candidiasis include isolation of *Candida* species from blood cultures, serological tests for antibody to *Candida*, and direct detection of *Candida* antigens or *Candida* metabolites in serum.

L. de Repentigny et al. (1985), *J. Clin. Microbiol.*, vol. 21(6), pages 972-79, used enzyme immunoassay and gas-liquid chromatography to compare the concentrations of three metabolic markers for candidiasis in serum samples from normal blood donors and high risk patients with and without invasive candidiasis, and concluded that the best approach to diagnosis of invasive candidiasis entails obtaining blood cultures and carrying out serial assays for mannan in serum.

B.V. Kumar et al. (1985), *Infect. Immun.*, vol. 48(1), pages 806-12, showed that human antibodies in blood serum from patients with histoplasmosis reacted most frequently to antigens shared by three disparate fungi, *Histoplasma capsulatum*, *Candida albicans*, and *Saccharomyces cerevisiae*, while they reacted with low frequency to antigens specific for individual fungal species. Kumar et al. suggested that specific epitopes may be present as part of the shared antigens, and that monoclonal antibodies to, e.g., the mitochondrial fraction of *C. albicans* might be used for specific immunodiagnosis.

B.B. Magee et al. (1987), *J. Bacteriol.*, vol. 169(4), pages 1639-43, found differences in patterns of restriction fragment length polymorphisms ("RFLPs") for the ribosomal DNA ("rDNA") regions from clinical isolates of various species and strains of *Candida*, and suggested that RFLPs may be clinically useful in biotyping various strains of *C. albicans* or in distinguishing various species.

L. Nelles et al. (1984), *Nucleic Acids Research*, vol. 12(23), pages 8749-8768, proposed an alignment of fourteen small ribosomal subunit RNA ("srRNA") sequences of eukaryotic, archaeobacterial, eubacterial, chloroplastic, and plant mitochondrial origin, on the basis of the presence of conserved features in the sequences, such as regions of theoretical secondary structure formation. They identified a number of presumed double-stranded regions ("proposed helices"), and which were present in all the aligned sequences (that is, generally conserved "universal helices"), regions which occurred only in eukaryotic srRNAs (conserved among eukaryotes; "eukaryote-specific helices"), and other regions which were found only in prokaryotic srRNAs (conserved among prokaryotes; "prokaryote-specific helices"). Nelles et al. proposed a system for numbering the corresponding regions of the aligned sequences. For example, a presumed universal

helix, numbered 19 in the Nelles alignment, is at bases having nucleotide numbers 632 through 641 (A. salina-specific nucleotide numbering) in each of six eukaryotes in the alignment. They also identified "variable areas," in which the primary as well as the secondary structure appeared to be especially variable among the srRNAs, including, for example, "area V4" (bases 642-870).

Within V4 is a long eukaryotic region (bases 642-805) having low sequence conservation. This region is entirely absent from all the other aligned srRNA sequences from archaeobacterial, prokaryotic, eukaryotic mitochondrial, and eukaryotic chloroplast sources.

D.E. Kohne, Canadian Pat. No. 1 215 904, describes a probe which is complementary to rRNA from any bacteria but is not complementary to human cell rRNA, for detecting the presence of *Mollicutes* species in human cell culture and other types of bacterial cells; and a method for making the probe. Kohne uses whole *Mollicutes hominis* rRNA as a template for reverse transcriptase synthesis of radioactive cDNA; hybridizes the cDNA to *M. hominis* rRNA to ensure that the cDNA is indeed complementary to this rRNA; then the labeled cDNA is hybridized with an excess of human rRNA, and the fraction not hybridizing to human rRNA is recovered to provide the probe. Kohne also describes using this method, beginning with *Trypanosoma brucei* rRNA, to select a cDNA probe that is complementary to trypanosome rRNA but is not complementary to human rRNA, for detection and quantitation of trypanosomes in human samples by hybridization. Kohne also suggests comparing known nucleotide sequences of rRNA from different organisms to identify "group specific sequences similar to a specific group of organisms", and making as a probe a sequence complementary to such a sequence.

In general, the invention features, in one aspect, a method for constructing a cDNA probe for use in detecting in a sample under conditions of predetermined stringency a target organism belonging to a strain of fungal microorganisms and not detecting a reference microorganism and not detecting any prokaryotic microorganism. The method includes steps of: determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from the target organism; comparing the determined nucleotide base region in the variable region from the target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from the reference microorganism, and selecting as a useful probe site a subsequence within said determined sequence; and synthesizing a cDNA complementary to the useful probe site, the cDNA being the probe.

The term "eukaryotic microorganisms", as that term is used herein, includes, for example and without limitation, fungi, protozoa, and mammalian cells; the term does not include chlamydia, mycoplasma and the like organisms, such as archaebac-

teria, intermediate between the conventional eukaryotes and prokaryotes. The term "fungal microorganisms", as that term is used herein, includes, for example and without limitation, molds and yeasts.

The term "variable region", as used herein, is a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and that has no corresponding region in prokaryotes. "Corresponding regions", as used herein, are regions of srRNA sequences which, when aligned as in the alignment proposed by Nelles et al., have the same positional relationships with respect to conserved regions, particularly with respect to proposed secondary structures.

In preferred embodiments, the variable region includes at least a part of the region immediately adjacent the 3' end of universal helix 19, that is, it includes at least a part of the region whose bases are assigned numbers 642 through 805 (*A. salina*-specific numbering) in the Nelles et al. alignment; the target organism is a strain of yeast, particularly a strain of yeast pathogenic to humans, more particularly a strain of *Candida*, and still more particularly a strain of *Candida albicans*; and the probe has a nucleotide base sequence including at least about 15 consecutive nucleotides selected from the sequence

5' GGAAAGGCTCGGCTGGGTCCAGTACGCAT-
CAAAAAGATGGACC 3'.

The invention features, in another aspect, an iterative method for constructing a cDNA probe for use in detecting in a sample a target organism belonging to a strain of fungal microorganisms and not detecting any of a plurality of reference microorganisms and probe not detecting any prokaryotic microorganism. The iterative method includes steps of: constructing a cDNA as summarized above and testing it for hybridization with RNA from the reference organisms to determine whether it has the desired specificity; if so, then the cDNA is the probe; but if not, then a refined probe is made by determining the base sequence in the variable region of srRNA from a next one of the reference microorganisms; comparing it with the corresponding base sequences already determined and selecting as a refined useful probe site a subsequence within the determined sequence, and synthesizing a refined cDNA complementary to the refined useful probe site; and testing it for hybridization with RNA from the reference organisms to determine whether it has the desired specificity. If so, then this first refined cDNA is the probe; but if not, the steps are repeated using a next one of the reference microorganisms, until a probe of the desired specificity is obtained.

The method of the invention provides diagnostic probes having as broad a specificity as is desired for a particular use; probes for detecting particular strains or species of fungal microorganisms can be obtained, as well as probes useful for screening groups of strains or species of fungal microorganisms.

In another aspect, the invention features a nucleic acid probe that detects, under conditions of predetermined stringency, the presence in a sample of

a first predetermined strain of fungal microorganism and fails to detect under those conditions the presence in the sample both of a second predetermined strain of fungal microorganism and of any prokaryotic organism. The probe includes a nucleic acid sequence capable of hybridizing, under those conditions, with a subsequence of nucleotide bases including a portion of a nucleotide base sequence of said first predetermined strain, and failing to hybridize, under those conditions, with a second subsequence of nucleotide bases including a corresponding portion of a nucleotide base sequence of the second predetermined strain. The corresponding nucleotide base sequences from the two strains correspond to a variable region that is present in eukaryotic small subunit ribosomal RNA and is not present in prokaryotic small subunit ribosomal RNA.

In another aspect, the invention features a method for detecting in a sample an organism belonging to a strain of fungal microorganisms, the method including providing a probe of the invention, deriving RNA from the sample, contacting the probe with the RNA under conditions that allow the probe to hybridize with small subunit rRNA to form hybrid nucleic acid complexes, and detecting the hybrid nucleic acid complexes as an indication of the presence in the sample of the organism. In another aspect, the invention features a kit, including a probe of the invention, and instructions for its use.

The probes of the invention can be used to detect both viable and non-viable organisms in pure or mixed culture, or directly without culturing in complex samples such as clinical samples or food-related material. The diagnostic test using these probes can be highly sensitive, because the srRNA target molecules are present in high numbers in each cell.

Preferred embodiments of the invention will now be described with reference to the accompanying drawings, in which:

Fig. 1 is a portion of the sequence alignment of srRNAs from a variety of organisms, as proposed by L. Nelles et al. (1984), *Nucleic Acid Research*, vol. 12(23), pages 8749-68.

Fig. 2 is a *Candida albicans* srRNA cDNA sequence, as determined by direct RNA sequencing.

Fig. 2A is an RNA sequence deduced from a portion of the cDNA sequence in Fig. 2, aligned with a corresponding portion shown in Nelles et al. (1984) of the *Saccharomyces cerevisiae* srRNA sequence.

Fig. 2B is the sequence of a synthetic oligomeric cDNA probe complementary to a useful probe site of the RNA sequence shown between arrows in Fig. 2A.

The abbreviations used in the figures are as follows: A, adenine; T, thymine; G, guanine; C, cytosine; U, uracil; M, either cytosine or adenine; R, either guanine or adenine; K, either guanine or thymine; a, adenine (uncertain); t, thymine (uncertain); A.S., *Artemia salina*; X.L., *Xenopus laevis*; R.n., *Rattus norvegicus*; S.c., *Saccharomyces cerevisiae*; Z.m. [upper], *Zea mays*; D.d., *Dictyostellum discoideum*; H.V., *Halobacterium volcanii*; E.c., *Escheri-*

chia coli; P.v., *Proteus vulgaris*; A.n., *Anacystis nidulans*; E.g., *Euglena gracilis* chloroplast; C.r., *Chlamydomonas reinhardtii* chloroplast; Z.m. [lower], *Zea mays* chloroplast; T.a., *Triticum aestivum* mitochondrion; In figure 1, smaller numbers are nucleotide numbers specific for *A. salina*, *H. volcanii*, *E. coli*, and *T. aestivum* mitochondria srRNA, and are placed above those sequences. The small numbers at the bottom refer to a general numbering system for this alignment. All nucleotide numbers in this application refer only to the *A. salina*-specific numbering appearing at the top. Labeled boxes identify sequences proposed by Nelles et al. as representing regions of common potential secondary structure.

Method of Constructing the Probe

Generally, a probe for detecting a particular eukaryotic organism ("target" organism) is made according to the invention by determining the sequence of nucleotide bases in a region ("variable region") of the srRNA from the target organism that is poorly conserved among eukaryotes and that has no corresponding region in prokaryotes; comparing that determined sequence with the corresponding known sequence from a reference organism whose presence is not to be detected; identifying for use as a probe target sequence ("useful probe site") a subsequence within the determined sequence that is substantially different from the corresponding subsequence in the reference organism; and synthesizing for use as the probe a single-stranded cDNA complementary to the identified probe target sequence. A detailed protocol for carrying out the method follows. One skilled in the art will appreciate that variation in the particulars of the protocol can be made without departing from the invention.

1) Determination of an rRNA sequence of the target organism in a region that is poorly conserved among eukaryotes and that has no corresponding region in prokaryotes ("variable region").

With reference now to Fig. 1, showing a part of the alignment scheme proposed by Nelles et al. (1984), the nucleotide base sequences of the srRNAs from a variety of eukaryotic organisms have a highly variable region, encountered after (i.e., toward the 3' end of the srRNA, and having higher sequence alignment numbers in the Nelles et al. proposed alignment) universal helix 19, for which there is no corresponding region in prokaryotic organisms. The bases in this region are assigned alignment numbers 642 through 805 in the Nelles et al. scheme, and the region is termed herein the "642-805 region".

A modification of the sequencing technique described by Lane et al. (1985) *Proc. Natl. Acad. Sci. USA*, vol. 82, pages 6955-6959, is employed to determine the srRNA sequence of the particular eukaryotic organism to be detected (the "target organism") in the region between bases numbered 642 and 805 in the Nelles et al. scheme (the "642-805 region"). A 17-base DNA oligomer (5' TTGGCAAATGCTTTCGC 3'), which is complementary to a strongly (although not completely) conserved eukaryotic region, that is, bases numbered

947 through 963 in the Nelles et al. scheme, wavy line, Fig. 1 (the "947-963 region"), was used as a specific primer for sequencing the region of interest by reverse transcriptase catalyzed primer extension. When hybridized with a preparation of total cellular RNA from the target organism whose rRNA is to be sequenced, the 17-mer anneals to the site at the 947-963 region on the target organism 18S rRNA. The primer fails to anneal to any site on any contaminating prokaryotic (16S) rRNA under comparable stringency conditions. The total cellular RNA fraction may be prepared in any of several ways, but preferably it is prepared by subjecting cell homogenates from the organism to several phenol extractions and several chloroform extractions, followed by ethanol precipitation, NaCl precipitation, and dissolution in water or an aqueous buffer.

Then, cDNA is synthesized as described, for example, in Lane et al., supra, by primer extension catalyzed by AMV reverse transcriptase using mixtures of deoxyribonucleoside triphosphates (dNTP's) and di-deoxyribonucleoside triphosphates (ddNTP's). The cDNA sequence is read directly from gels showing the lengths of primer-extended products in which synthesis has been terminated by incorporation of a ddNTP precursor.

Primers other than the 947-963 region 17-mer also can be used: for example, the region including bases numbered 910 through 926 in the Nelles et al. scheme (the "910-926 region") is conserved among 5 of the 6 eukaryotes shown in Fig. 1 (5' GAGGUGAAAUUCUUGGA 3'), while that of slime mold is the same except for a G at base 922 and a U at base 924. Thus, an equimolar mixture of complementary 17-mers containing two 2-fold redundancies at these positions (5' TC(C/A)A(A/C)GAATTCACCTC 3') can also be useful as a primer for primer extension synthesis of the 642-805 region.

Then the sequence of bases in the variable region from the organism to be detected, that is, the corresponding 642-805 region, is deduced from the cDNA sequence. It is preferred to deduce the rRNA sequence from the sequenced cDNA rather than from sequenced genomic subclones for several reasons: a) it is much faster since it involves only a small number of steps; b) it is much more efficient in that it minimizes the number of bases sequenced outside the region of interest; c) it is more relevant to development of diagnostic DNA probes in that the cDNA sequence obtained accurately reflects the sequence of the mature 18S rRNA molecules in cells, after all processing of larger precursor molecules has been completed. Although rDNA cistrons tend to be highly amplified in eukaryotic genomes (e.g. in *Saccharomyces cerevisiae* over 100 direct tandem repeats at a single genomic locus, all of which are transcriptionally active), the rRNA transcription products will be several orders of magnitude more highly amplified. Moreover, with simple disruption procedures used to release rRNA target molecules in a diagnostic test, the relative number of rDNA genes (complexed with chromosomal proteins) released will be very small or nonexistent. Thus, if a certain species were to contain an atypical rDNA gene structure associated with unusual splice

sites, for example, then deducing rRNA sequence from sequenced genomic clones might lead to erroneous predictions of the mature 18S rRNA sequence.

2) Comparison of the variable region sequence with the corresponding sequences of one or more organisms not to be detected.

The deduced rRNA sequence of the organism to be detected is now aligned with the corresponding rRNA sequence of the strain or species whose presence is not to be detected by the probe.

For example (this example is described in detail below), in order to design a probe specific detecting the yeast pathogen *Candida albicans* and not detecting *Saccharomyces cerevisiae* or any prokaryotes the 642-805 region sequence from *C. albicans* organism is first obtained as described above and then aligned with the corresponding sequence from *Saccharomyces cerevisiae*. The Example below provides a detailed description of how the 642-805 region sequence of *Candida albicans* can be determined using the general method described above.

3) Analysis of the aligned sequence(s) to determine a subregion containing a useful probe site ("UPS").

The choice of a UPS is made by inspecting the aligned sequences for mismatched bases. Generally the object is to select a UPS such that similarities in the sequence between the organism to be detected and the corresponding portions of the sequence in closely related species (the reference organism) are minimized. Probes designed according to this principle maximize the relative thermal instability, under predetermined conditions of stringency, of hybrids formed by hybridization with the cDNA probe of RNAs from the species not to be detected. One way to accomplish this, for example, is to choose the UPS so that predicted mismatch bases in hybrids are distributed uniformly along the sequence or are clustered near the middle rather than near the ends of the UPS sequence.

4) Synthesis and labelling of a single-stranded oligomeric cDNA probe complementary to the UPS region of the target organism.

The cDNA probe complementary to the target organisms UPS region is preferably synthesized using an automated nucleotide synthesizer. Labelling of the cDNA probe may be performed after synthesis on an automated nucleotide synthesizer by methods well known in the art. For example, the probe can be made to carry a non-radioactive label by using specially modified dNTP precursors during the nucleotide synthesis, as described generally in Rashtchian et al. (1987), *Clinical Chemistry*, vol. 33(a), pp. 1526-30; and in *Applied Biosystems User Bulletin Number 38* (Nov. 3, 1986), pp. 1-5. Alternatively, unphosphorylated 5' ends can be labelled with ^{32}P following reaction with ^{32}P -gamma-ATP and polynucleotide kinase enzyme, according to conventional techniques as described generally in, for example, Maniatis et al. (1982), *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor,

New York.

Finally the probe is proven by testing it for specifically detecting in a sample the presence of the target organism and not detecting the organism whose presence is not to be detected.

Use

The cDNA probes of the invention are used to detect specific eukaryotic microorganisms, particularly *Candida* species of yeast and other fungi of importance to clinical testing or food testing, according to the following general steps:

1) Disruption of yeast cells or fungal hyphae.

The rRNA from organisms contained in the sample or in cultures derived from the sample is released from the cells by mechanical means, preferably by vigorous vortex mixing of the sample with acid-washed glass beads (about 0.45-0.50 mm diameter) as described generally in C.P. Kurtzman et al., (Review), In: J.F.T. Spencer et al., eds. (1983), *Yeast Genetics: Fundamental and Applied Aspects*, Springer-Verlag, New York, p. 140.

2) Denaturation of the rRNA.

The cellular homogenate is then treated in well-known fashion with denaturants or with heat (or both) to open up stem-loop or other secondary structures in the target rRNA.

3) Hybridization of sample with cDNA probe.

Then the sample is contacted with the cDNA probe under conditions of stringency in which the probe is known to hybridize with target rRNA. The denatured rRNA can be immobilized on nitrocellulose or nylon membranes and hybridized with the probe in solution. Alternatively, hybridization conditions in which both target and probe are in solution can be used.

4) Removal of non-hybridized probe.

For sample rRNA immobilized on membrane filters, non-hybridized probe can be removed by washing the filters in a solution of salt at a suitable concentration and at a suitable temperature according to well-known methods. For solution hybridizations, any method can be used, preferably a method employing an immobilized anti-DNA:RNA antibody, as described generally in Rashtchian et al., supra, to capture and immobilize RNA-DNA hybrid molecules while non-hybridized probe is removed by subsequent washes.

5) Detection of label in hybridized probe.

Probes labeled with radioactive isotopes can be detected in any of a number of ways, for example by some form of radioisotope counter or by exposure of photographic film. The signal from non-radioactive probes is detected using methods compatible with the type of labelling.

Many variations of the hybridization reaction are possible. For example, steps 1) and 2) can be combined by breaking cells directly in a buffer containing denaturants. For solution hybridizations, cells can be broken in a buffer that also contains the

labeled cDNA probe, followed by heat denaturation, then renaturation by cooling. In this case partial hydrolysis of the RNA with mild alkali or ribonuclease can enhance the accessibility of the probe to a site that might otherwise not be fully denatured.

Example

The following example demonstrates the manufacture of a *Candida*-specific rRNA probe according to the invention. Under selected stringency conditions the probe detects the presence of *Candida* species and does not detect other yeast species or other fungi or prokaryotes. The example is given by way of illustration and is not intended to limit the claims.

The probe was designed and made generally as described above, based on analysis of 18s rRNA sequence data from a strain of *Candida albicans*. The specificity of the probe was assayed against a variety of organisms, including other *Candida albicans* strains, *Candida* species, and less closely related yeasts and fungi. The organisms used in the specificity assay are listed in Table 1 ("Series A": *Candida* strains, *Listeria monocytogenes*, *E. coli*) and Table 2 ("Series B": other yeasts and fungi; human white blood cells).

TABLE 1
STRAINS (SERIES A)

	ORGA- NISM	STRAIN	OTHER NAMES
5	1 <i>Candida albicans</i>	ATCC 18804*	
10	2 <i>Candida albicans</i>	ATCC 14053	
	3 <i>Candida albicans</i>	ATCC 11006	<i>Candida stellatoidea</i>
15	4 <i>Candida glabrata</i>	ATCC 2001*	<i>Torulopsis glabrata</i> <i>Cryptococcus</i> "
20	5 <i>Candida tropicalis</i>	ATCC 750*	
	6 <i>Candida pseudotropicalis</i>	ATCC 4135*	<i>Candida kefyr</i>
25			<i>Kluyveromyces fragilis</i> "
30	7 <i>Candida krusei</i>	ATCC 6258*	<i>marxianus</i> <i>Issatchenkia orientalis</i>
35	8 <i>Candida parapsilosis</i>	ATCC 22019*	<i>Monilia parapsilosis</i>
40	9 <i>Candida guilliermondii</i>	ATCC 6260*	<i>Pichia guilliermondii</i>
	10 <i>Candida lusitanae</i>	ATCC 42720	<i>Clavispora lusitanae</i>
45	11 <i>E. coli</i>	YMC	
	12 <i>Listeria monocytogenes</i>	SLCC 6933, SLCC 6835	

* Type culture

TABLE 2
STRAINS (SERIES B)

	ORGA- NISM	STRAIN	OTHER NAMES
1	<u>Saccharo- myces cerevisiae</u>	X2180-1A	
2	<u>Schizo- saccharo- myces pombe</u>	ade6	
3	<u>Schwan- niomyces castellii</u>	ATCC 26076	
4	<u>Yarrowia lipolytica</u>	ATCC 32338	<u>Candida lipolytica</u> <u>Saccharo- mycopsis lipolytica</u>
5	<u>Saccharo- myces uvarum</u>	comm. beer	<u>Saccharo- myces carlsber- gensis</u>
6	<u>Trichos- poron capitatum</u>	ATCC 10663	<u>Geotri- chum capitatum</u> <u>Blasto- schizo- myces capitatus</u>
7	<u>Trichos- poron beigelii</u>	ATCC 28592*	<u>Geotri- choides cutaneus</u> <u>Trichos- poron cutaneum</u>
8	<u>Trichophy- ton mentagro- phytes</u>	ATCC 28185	<u>Arth- roderma benha- miae</u>
9	<u>Trichophy- ton rubrum</u>	ATCC 28188	
10	<u>Alternaria alternata</u>	ATCC 13963	tomato black mold
11	Human White Blood Cells		

*Type culture

1) Preparation of *Candida albicans* total RNA.

Candida albicans (ATCC strain 18804) yeast cells exponentially growing at 30°C in 50 ml of liquid YEPD medium (1% yeast extract, 2% peptone, 2% dextrose) were distilled water-washed by low-speed centrifugation, suspended in 3 ml of an RNA extraction buffer (0.1 M LiCl, 0.1 M EDTA, 0.1 M

Tris-HCl, pH 7.4), and placed on ice. Two grams of acid-washed and distilled water-rinsed glass beads (0.45-0.50 mm diameter) were added and the mixture repeatedly shaken at the highest speed on a vortex mixer for 10 sec, and then placed in an ice bath for 10 sec, until a total time of 2 min had elapsed. Then, 0.05 ml of 10% sodium dodecylsul-
fate (SDS) and 1 ml of phenol (equilibrated with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added and the mixture agitated briefly. Following low-speed centrifugation to separate the phases, the top aqueous phase was removed and twice again extracted with phenol, then twice extracted with chloroform-isoamyl alcohol (24:1) and then adjusted to 0.3 M sodium acetate. Total nucleic acids (RNA and small plasmid DNA) were precipitated with 2 volumes of 100% ethanol. The precipitates were washed once with 70% ethanol and once with 100% ethanol, dried under vacuum, dissolved in sterile distilled water, and stored at -20°C. High molecular weight RNA was further separated from DNA and from low molecular weight RNA by precipitation with 2 M NaCl.

The resulting solution was diluted in distilled water and the UV absorption measured at 260 nm. Concentration of nucleic acid was determined on the basis of $A_{260} = 1.0$ (1-cm path length) for 50 micrograms per ml. Subsequent analysis by agarose gel electrophoresis revealed that greater than 99% of the nucleic acid stained by ethidium bromide is composed of a mixture of 18S and 25S subunit rRNAs.

2) Obtaining a *Candida albicans* srRNA cDNA sequence.

A cDNA sequence was obtained by generally following the primer-extension method described in Lane et al. (1985) *Proc. Natl. Acad. Sci. USA*, vol. 82, pages 6955-6959, using as a template the *Candida albicans* total RNA preparation described above and using as a primer the synthetic oligomeric DNA sequence 5' TTGGCAAATGCTTTCGC 3' (Fig. 1, wavy line). The cDNA sequence obtained is shown in Fig. 2.

3) Identification of a possible useful probe site ("UPS") and synthesis of a cDNA probe.

The deduced RNA sequence of the *C. albicans* srRNA in the 642-805 region then was compared with the corresponding region, shown in Nelles et al. (1984), from *Saccharomyces cerevisiae*, by aligning the sequences as shown in Fig. 2A.

One possible UPS, shown between arrows in Fig. 2A, was chosen by inspection of the aligned sequences. A 43-nucleotide oligomeric DNA complementary to this UPS (shown in Fig. 2B) was synthesized on an automated nucleotide synthesizer. The 43-mer was gel-purified and labeled at the 5' end with ^{32}P , following reaction with ^{32}P -gamma-ATP and T4 polynucleotide kinase enzyme. The end-labeled 43-mer *C. albicans* rRNA probe was gel-purified and assayed for specificity by hybridization reactions with various total RNA preparations from other eukaryotic organisms.

4) Assay for probe specificity.

The specificity of the 43-mer probe was tested empirically by first preparing total RNA from a collection of organisms (Tables 1 and 2) using methods the same as or similar to that described above for *C. albicans*. RNAs were denatured by dilution into a solution containing final concentrations of 7.5% formaldehyde and 10X SSC (1X SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), followed by heating to 50°C for 15 min., then quick cooling by immersion in an ice bath. Equal amounts (approx. 1 microgram) of denatured RNA from each strain were immobilized on 2 different nylon membrane filters. One filter was hybridized with the labeled 43-mer test probe, while the other was hybridized with a similarly end-labeled oligomeric DNA probe. This latter probe is an equimolar mixture of 4 different 18-mers that are complementary to the 572-589 region (region A, Fig. 1): 5' G(A/T)ATTACCGCGGC(G/T)GCTG 3'. This latter probe is used here as a control for the amount of denatured 18S rRNA immobilized on the filters because this region is very strongly conserved among small subunit rRNAs of all organisms (Lane et al. (1985) have described these 18-mers as "universal primers" for use in the cDNA primer-extension sequencing technique).

Filters were hybridized overnight (greater than 16 hours) under low-stringency conditions at room temperature in a simplified cocktail containing 1 mg/ml salmon sperm DNA (sonicated and heat-denatured), 0.5% SDS, 6X SSC, then subjected to low-stringency (6X SSC, 37°C) or high-stringency (0.1X SSC, 65°C) washes. The assay for hybridization was by exposure to X-ray film.

The 43-mer probe detected strains of only 3 other *Candida* species, besides the *Candida albicans* controls: *Candida tropicalis*, *Candida parapsilosis*, and *Candida guilliermondii* (weak positive). Following the initial low-stringency wash, all other strains in this collection (Tables 1 and 2) were essentially negative or only very slightly positive with the 43-mer probe. Following a high-stringency wash, the same pattern of hybridization obtained; the strains showing slight positives after low stringency show negative under high stringency. Results with the control 18-mers showed that all samples hybridized approximately equally well, showing that approximately equal amounts of hybridizable RNA were present on the filter.

Regarding the detection of *Candida* species and other unrelated yeasts and fungi comprising our test panel, the desirable negative test-probe results indicate a lack of hybridization rather than a lack of RNA, because in such cases the results of the positive control probe was positive. This indicates that hybridization took place and hence that hybridizable RNA was present. The sample derived from human white blood cells contains mostly DNA (only a small fraction is 18S and 28S rRNA). This is consistent with the observed results wherein the test probe did not hybridize but the positive control probe did, albeit weakly.

5) Assay for greater specificity under higher stringency.

Candida strains A1-A9 (Series A; Table 1) were examined by hybridization analysis with the 43-mer test probe under higher stringency conditions than those employed as described in section 4, above. For each strain, both a purified total RNA sample and a cell suspension sample were tested. The RNA sample was prepared as described above. Cells were suspended in distilled water and adjusted to a uniform turbidity corresponding to absorbance at 600 nm of 0.5. Equal aliquots of 0.3 ml of the cell suspension were adjusted to contain final concentrations of 10X SSC and 7.5% formaldehyde in a final volume of 1.0 ml, in 13mm x 100mm glass culture tubes. Two grams of glass beads were added and the tubes containing the mixture were placed in an ice bath, shaken at high speed on a vortex mixer for 30 seconds, incubated at 50°C for 15 min, and quickly cooled in an ice bath. 0.2 ml of the resulting homogenate (free of glass beads, which had quickly settled out) was immobilized on a nylon membrane filter and hybridized for 90 minutes with the labeled 43-mer *C. albicans* rRNA probe in 6X SSC at 65°C. The filters were then washed in 6X SSC at 65°C and assayed by exposure to X-ray film. The filters were also subsequently washed in 0.1X SSC at 65°C and assayed by exposure to X-ray film.

Under these conditions, *Candida tropicalis* was detected by the *C. albicans* rRNA-derived probe following the 6X SSC wash but not following the 0.1X SSC wash (approximately 99% of the radioactivity was lost in the high-stringency wash). Following the 0.1X SSC wash, *Candida parapsilosis* showed strong positive, while *Candida guilliermondii* showed weak positive. Moreover, positive signals were obtained from samples derived from whole yeast cells only in cases where the purified RNA samples are also strongly positive. *Candida* strains (Table 1) not detected with the 43-mer probe are negative for both purified RNA and whole-cell samples.

This *C. albicans*-derived srRNA 43-mer probe is thus demonstrably useful in a diagnostic assay for the presence of *Candida* yeast cells. The probe failed to detect non-*Candida* yeasts and other unrelated fungi or prokaryotic organisms (section 4 above), while at the same time detecting all tested *Candida* species known as actual or potential human pathogens, with the exceptions of *Candida glabrata*, *Candida krusei*, *Candida kefyr*, and *Candida lusitanae*. In addition, it detected *C. parapsilosis* and *C. guilliermondii*. Determination and alignment of sequences for *Candida parapsilosis* and *Candida guilliermondii*, using the general method described above, can provide both more specific probes and more general probes, including *Candida*-specific, yeast-specific, and fungi-specific probes.

Total RNA samples (5 micrograms each) from strains A1-A7 (*Candida* species), A11 (*E. coli*), B1 (*Saccharomyces cerevisiae*), B6 (*Trichosporon capitatum*), and B9 (*Trichophyton rubrum*) were denatured with formaldehyde and heat and then analyzed by electrophoresis through a 1.2% agarose gel containing formaldehyde. Ethidium bromide staining confirmed that all samples contained ap-

proximately equal abundances of major rRNA subunits (i.e. approximately uniform staining of 16S, 18S, 23S, and 25S bands). Following Northern blot transfer and immobilization on a nitrocellulose membrane the filter was probed with the *C. albicans*-derived 43-mer under the same hybridization and wash conditions as described above. Single bands of strongly positive hybridization were observed only in *Candida albicans* strains A1-A3 at a position corresponding to the migration of the ethidium bromide-stained 18S rRNA subunit. The 25S subunits exhibited no hybridization whatsoever, nor was there any hybridization detectable anywhere else throughout the fractionated total RNA samples. The 18S subunit from strain A5 (*Candida tropicalis*) exhibited a weaker hybridization signal than that from the other three strains. Fractionated total RNAs from all the other samples failed to exhibit any hybridization with the 43-mer probe. This example shows that positive hybridization exhibited by these total RNA samples was due to presence of 18S rRNA sequences homologous with and complementary to the *C. albicans*-derived 43-mer srRNA cDNA probe.

6) Further refinement of probe specificity.

The method of the invention can be used to make a more narrowly specific *Candida albicans* probe, that is, a probe that detects *C. albicans* and does not detect *C. parapsilosis* or *C. guilliermondii*, or to make a probe that detects a selected group of species, such as, for example, selected known actual or potential human pathogens, including *C. glabrata*, *C. krusei*, *C. kefyr*, and *C. lusitaniae*.

Making a more narrowly specific probe requires elimination of "false positives". In the above example, detection of *C. parapsilosis* and *C. guilliermondii* can be eliminated by determining the variable region base sequence of the srRNA from one of the species, preferably *C. parapsilosis*, the more strongly positive one, aligning the base sequence with known variable region sequences, identifying a potential UPS by inspection of the alignments, and then synthesizing and testing a cDNA to the UPS. The potential UPS can be chosen as one which would hybridize to *C. albicans* but not to *C. parapsilosis* or to other species whose variable region sequences are known and included in the alignment. False positives, if any, detected in the test of this refined probe can be eliminated by iteration of the above steps. Only variable region sequences of particular false positives need be determined, and elimination of one such false positive can result in elimination as well of one or more others, so that not all sequences need be determined. A tremendous savings of steps and resources can thereby be realized.

Making a probe that detects a selected broader group of strains or species amounts to eliminating "false negatives". In the above example, detection of *C. glabrata*, *C. krusei*, *C. kefyr*, and *C. lusitaniae* can be obtained by determining the variable region base sequence of one of these species, for example, *C. glabrata*, aligning the base sequence with known variable region sequences, identifying a known UPS

by inspection of the alignments, and then synthesizing and testing a cDNA to the UPS. The potential UPS can be chosen as one complementary to a DNA probe that would hybridize with both *C. albicans* and *C. glabrata*, but not with other species whose variable region sequences are known and included in the alignment.

False negatives, if any, detected in the test of this refined probe can be eliminated by iteration of the above steps. As is the case with refinement to eliminate false positives, elimination of one false negative can result in elimination as well of one or more others, so that here, too, not all sequences need be determined.

In this manner, a probe having a desired breadth of specificity for a particular species or strain or group of species or strains of fungal microorganisms can be made for any clinical or food-related setting.

Other Embodiments

Other embodiments are within the following claims. For example, probes can be made and used according to the invention for fungal microorganisms other than *Candida albicans*. Of particular interest are other fungal pathogens of humans, including, for example, yeast-like pathogens such as species of *Geotrichum*; non-yeast dermatophytes such as species of *Trichophyton*, *Microsporum*, and *Epidermophyton*; and systemic and non-systemic yeast pathogens such as species of *Cryptococcus*, *Coccidioides*, *Histoplasma*, *Blastomyces*, and other species of *Candida*.

Claims

1. A method for constructing a cDNA probe for use in detecting in a sample under conditions of predetermined stringency a target organism belonging to a strain of fungal microorganisms and not detecting in said sample under said conditions a reference microorganism, said probe not detecting in said sample under said conditions any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from said target organism, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and having no corresponding region in prokaryotes,

(2) comparing said nucleotide base region in said variable region from said target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said reference microorganism, and selecting as a useful probe site a subsequence within said determined sequence, and

(3) synthesizing a cDNA complementary to said useful probe site, said cDNA being

said probe.

2. A method for constructing a cDNA probe for use in detecting in a sample a target organism belonging to a strain of fungal microorganisms and not detecting any of a plurality of reference microorganisms, said probe not detecting any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from said target organism, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and said variable region having no corresponding region in prokaryotes,

(2) comparing said nucleotide base region in said variable region from said target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from one of said reference microorganisms, and selecting as a potential probe site a subsequence within said determined sequence.

(3) synthesizing a cDNA complementary to said useful probe site,

(4) contacting said cDNA with RNA from said reference microorganisms under conditions that allow said cDNA to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(5) determining whether hybrid nucleic acid complexes are formed during said contacting, and

(6) if hybrid nucleic acid complexes are not formed during said contacting, then said cDNA is said probe; but

(7) if hybrid nucleic acid complexes are formed during said contacting, then

(a) determining a nucleotide base sequence in said variable region of small subunit ribosomal RNA from a next one of said reference organisms,

(b) comparing said nucleotide base region in said variable region from said next reference organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said first reference microorganism and with the corresponding region of small subunit ribosomal RNA from said target microorganism and selecting as a potential next refined probe site a subsequence within said determined sequence,

(c) synthesizing a cDNA complementary to said next refined probe site,

(d) contacting said cDNA complementary to said next refined probe site with RNA from said reference microorganisms under conditions that allow said cDNA complementary to said next refined probe site to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(e) determining whether hybrid nucleic acid complexes are formed during said

contacting, and

(f) if hybrid nucleic acid complexes are not formed during said contacting, then said cDNA complementary to said next refined probe site is said probe; but

(g) if hybrid nucleic acid complexes are not formed during said contacting, then

(h) repeating steps (a) through (g).

3. A method for constructing a cDNA probe for use in detecting in a sample a target organism belonging to any of a plurality of predetermined strains of fungal microorganisms and not detecting any of a plurality of reference microorganisms, said probe not detecting any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from a first one of said predetermined strains of fungal microorganisms, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and said variable region having no corresponding region in prokaryotes,

(2) comparing said nucleotide base region in said variable region from said first strain with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from one of said reference microorganisms, and selecting as a potential probe site a subsequence within said determined sequence,

(3) synthesizing a cDNA complementary to said useful probe site,

(4) contacting said cDNA with RNA from said fungal microorganisms and with RNA from said reference microorganisms under conditions that allow said cDNA to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(5) determining whether hybrid nucleic acid complexes are formed with RNA from said predetermined strain of fungal microorganisms during said contacting and determining whether hybrid nucleic acid complexes are formed with RNA from said reference microorganisms during said contacting, and

(6) if hybrid nucleic acid complexes are formed with RNA from each of said predetermined strains during said contacting and are not formed with RNA from any of said reference microorganisms during said contacting, then said cDNA is said probe; but

(7) if hybrid nucleic acid complexes are not formed with RNA from any of said reference microorganisms and if hybrid nucleic acid complexes are not formed with RNA from one or more of said predetermined strains during said contacting, then

(a) determining a nucleotide base sequence in said variable region of small subunit ribosomal RNA from a next one of

said predetermined strains,

(b) comparing said nucleotide base region in said variable region from said next predetermined strain with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said first predetermined strain and the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said one of said reference microorganisms, and selecting as a potential next refined probe site a subsequence within said determined sequence,

(c) synthesizing a cDNA complementary to said next refined probe site,

(d) contacting said cDNA complementary to said next refined probe site with RNA from said fungal microorganisms and with RNA from said reference microorganisms under conditions that allow said cDNA complementary to said next refined probe site to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

e) determining whether hybrid nucleic acid complexes are formed with RNA from said fungal microorganisms during said contacting and determining whether hybrid nucleic acid complexes are formed with RNA from said reference microorganisms during said contacting, and

(f) if hybrid nucleic acid complexes are formed with RNA from each of said predetermined strains and are not formed with RNA from any of said reference microorganisms during said contacting, then said cDNA is said probe; but

(g) if hybrid nucleic acid complexes are not formed with RNA from any of said reference microorganisms and if hybrid nucleic acid complexes are not formed with RNA from one or more of said predetermined strains during said contacting then

(h) repeating steps (a) through (g).

4. A method as claimed in claim 1, 2 or 3 wherein said variable region corresponds to the variable region adjacent the 3' end of universal helix 19.

5. A method as claimed in claim 1, 2 or 3 wherein said variable region corresponds to the 642-805 region.

6. A nucleic acid probe that detects, under conditions of predetermined stringency, the presence in a sample of a first predetermined strain of fungal microorganism and fails to detect, under said conditions, the presence in said sample both of a second predetermined strain of fungal microorganism and of any prokaryotic organism, said probe comprising a nucleic-acid sequence capable of hybridizing, under said conditions, with a subsequence of nucleotide bases comprising a portion of a nucleotide base sequence of said first predetermined strain, said nucleic acid sequence failing to hybridize,

under said conditions, with a second subsequence of nucleotide bases comprising a corresponding portion of a nucleotide base sequence of said second predetermined strain, said nucleotide base sequence of said first predetermined strain and said corresponding nucleotide base sequence from said second predetermined second strain each corresponding to a variable region that is present in eukaryotic small subunit ribosomal RNA and is not present in prokaryotic small subunit ribosomal RNA.

7. A probe as claimed in claim 6, wherein said first predetermined strain is a strain of yeast, for example which is pathogenic to humans.

8. A probe as claimed in claim 6, wherein said first predetermined strain is a strain of *Candida*, preferably *Candida albicans*.

9. A probe as claimed in claim 6 wherein said first predetermined strain is *Candida albicans* ATCC strain 18804, and said second predetermined strain is a strain of *Saccharomyces cerevisiae*.

10. A probe as claimed in any one of claims 6 to 9, wherein said variable region is immediately adjacent the 3' end of universal helix 19.

11. A probe as claimed in any one of claims 6 to 9, wherein said variable region is the 642-805 region.

12. A nucleic acid probe capable of detecting in a sample, under predetermined stringency conditions, *Candida albicans*, said probe comprising a nucleotide base sequence of at least about 15 consecutive nucleotides selected from the sequence

5' GGAAAGGCTCGGCTGGGTCCAGTACG-CATCAAAAAGATGGACC 3'.

13. A method for detecting in a sample an organism belonging to a strain of eukaryotic microorganisms, comprising

providing a probe as claimed in any one of claims 6 to 12,

deriving RNA from said sample,

contacting said probe with said RNA under conditions that allow said probe to hybridize with small subunit rRNA to form hybrid nucleic acid complexes, and

detecting said hybrid nucleic acid complexes as an indication of the presence in said sample of said organism.

14. A kit, comprising a probe as claimed in any one of claims 6 to 12 and instructions for its use.

Claims for the following Contracting State: ES

1. A method for constructing a cDNA probe for use in detecting in a sample under conditions of predetermined stringency a target organism belonging to a strain of fungal microorganisms and not detecting in said sample under said conditions a reference microorganism, said probe not detecting in said sample under said conditions any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from said target organism, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and having no corresponding region in prokaryotes,

(2) comparing said nucleotide base region in said variable region from said target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said reference microorganism, and selecting as a useful probe site a subsequence within said determined sequence, and

(3) synthesizing a cDNA complementary to said useful probe site, said cDNA being said probe.

2. A method for constructing a cDNA probe for use in detecting in a sample a target organism belonging to a strain of fungal microorganisms and not detecting any of a plurality of reference microorganisms, said probe not detecting any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from said target organism, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and said variable region having no corresponding region in prokaryotes,

(2) comparing said nucleotide base region in said variable region from said target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from one of said reference microorganisms, and selecting as a potential probe site a subsequence within said determined sequence,

(3) synthesizing a cDNA complementary to said useful probe site,

(4) contacting said cDNA with RNA from said reference microorganisms under conditions that allow said cDNA to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(5) determining whether hybrid nucleic acid complexes are formed during said contacting, and

(6) if hybrid nucleic acid complexes are not formed during said contacting, then said cDNA is said probe; but

(7) if hybrid nucleic acid complexes are formed during said contacting, then

(a) determining a nucleotide base sequence in said variable region of small subunit ribosomal RNA from a next one of said reference organisms,

(b) comparing said nucleotide base region in said variable region from said next reference organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said first reference microorganism and

with the corresponding region of small subunit ribosomal RNA from said target microorganism and selecting as a potential next refined probe site a subsequence within said determined sequence,

(c) synthesizing a cDNA complementary to said next refined probe site,

(d) contacting said cDNA complementary to said next refined probe site with RNA from said reference microorganisms under conditions that allow said cDNA complementary to said next refined probe site to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(e) determining whether hybrid nucleic acid complexes are formed during said contacting, and

(f) if hybrid nucleic acid complexes are not formed during said contacting, then said cDNA complementary to said next refined probe site is said probe; but

(g) if hybrid nucleic acid complexes are not formed during said contacting, then

(h) repeating steps (a) through (g).

3. A method for constructing a cDNA probe for use in detecting in a sample a target organism belonging to any of a plurality of predetermined strains of fungal microorganisms and not detecting any of a plurality of reference microorganisms, said probe not detecting any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from a first one of said predetermined strains of fungal microorganisms, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and said variable region having no corresponding region in prokaryotes.

(2) comparing said nucleotide base region in said variable region from said first strain with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from one of said reference microorganisms, and selecting as a potential probe site a subsequence within said determined sequence,

(3) synthesizing a cDNA complementary to said useful probe site,

(4) contacting said cDNA with RNA from said fungal microorganisms and with RNA from said reference microorganisms under conditions that allow said cDNA to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(5) determining whether hybrid nucleic acid complexes are formed with RNA from said predetermined strain of fungal microorganisms during said contacting and determining whether hybrid nucleic acid complexes are formed with RNA from said reference microorganisms during said contacting, and

(6) if hybrid nucleic acid complexes are formed with RNA from each of said predetermined strains during said contacting and are not formed with RNA from any of said reference microorganisms during said contacting, then said cDNA is said probe; but

(7) if hybrid nucleic acid complexes are not formed with RNA from any of said reference microorganisms and if hybrid nucleic acid complexes are not formed with RNA from one or more of said predetermined strains during said contacting, then

(a) determining a nucleotide base sequence in said variable region of small subunit ribosomal RNA from a next one of said predetermined strains,

(b) comparing said nucleotide base region in said variable region from said next predetermined strain with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said first predetermined strain and the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said one of said reference microorganisms, and selecting as a potential next refined probe site a subsequence within said determined sequence,

(c) synthesizing a cDNA complementary to said next refined probe site,

(d) contacting said cDNA complementary to said next refined probe site with RNA from said fungal microorganisms and with RNA from said reference microorganisms under conditions that allow said cDNA complementary to said next refined probe site to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(e) determining whether hybrid nucleic acid complexes are formed with RNA from said fungal microorganisms during said contacting and determining whether hybrid nucleic acid complexes are formed with RNA from said reference microorganisms during said contacting, and

(f) if hybrid nucleic acid complexes are formed with RNA from each of said predetermined strains and are not formed with RNA from any of said reference microorganisms during said contacting, then said cDNA is said probe; but

(g) if hybrid nucleic acid complexes are not formed with RNA from any of said reference microorganisms and if hybrid nucleic acid complexes are not formed with RNA from one or more of said predetermined strains during said contacting, then

(h) repeating steps (a) through (g).

4. A method as claimed in claim 1, 2 or 3 wherein said variable region corresponds to the variable region adjacent the 3' end of universal helix 19.

5. A method as claimed in claim 1, 2 or 3 wherein said variable region corresponds to the 642-805 region.

6. A process for preparing nucleic acid probe that detects, under conditions of predetermined stringency, the presence in a sample of a first predetermined strain of fungal microorganism and fails to detect, under said conditions, the presence in said sample both of a second predetermined strain of fungal microorganism and of any prokaryotic organism, said probe comprising

a nucleic-acid sequence capable of hybridizing, under said conditions, with a subsequence of nucleotide bases comprising a portion of a nucleotide base sequence of said first predetermined strain,

said nucleic acid sequence failing to hybridize, under said conditions, with a second subsequence of nucleotide bases comprising a corresponding portion of a nucleotide base sequence of said second predetermined strain, said nucleotide base sequence of said first predetermined strain and said corresponding nucleotide base sequence from said second predetermined strain each corresponding to a variable region that is present in eukaryotic small subunit ribosomal RNA and is not present in prokaryotic small subunit ribosomal RNA, the process comprising coupling successive nucleotides together and/or ligating oligo- and/or poly-nucleotides.

7. A process as claimed in claim 6, wherein said first predetermined strain is a strain of yeast, for example which is pathogenic to humans.

8. A process as claimed in claim 6, wherein said first predetermined strain is a strain of *Candida*, preferably *Candida albicans*.

9. A process as claimed in claim 6 wherein said first predetermined strain is *Candida albicans* ATCC strain 18804, and said second predetermined strain is a strain of *Saccharomyces cerevisiae*.

10. A process as claimed in any one of claims 6 to 9, wherein said variable region is immediately adjacent the 3' end of universal helix 19.

11. A process as claimed in any one of claims 6 to 9, wherein said variable region is the 642-805 region.

12. A process for the preparation of a nucleic acid probe capable of detecting in a sample, under predetermined stringency conditions, *Candida albicans*, said probe comprising a nucleotide base sequence of at least about 15 consecutive nucleotides selected from the sequence

5' GGAAAGGCTCGGCTGGGTCCAGTACG-CATCAAAAAGATGGACC 3', the process comprising coupling successive nucleotides together and/or ligating oligo-and/or poly-nucleotides.

13. A method for detecting in a sample an organism belonging to a strain of eukaryotic microorganisms, comprising

providing a probe preparable by a process as claimed in any one of claims 6 to 12,
deriving RNA from said sample,
contacting said probe with said RNA under conditions that allow said probe to hybridize with small subunit rRNA to form hybrid nucleic acid complexes, and

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detecting said hybrid nucleic acid complexes as an indication of the presence in said sample of said organism.

14. A kit, comprising a probe preparable by a process as claimed in any one of claims 6 to 12 and instructions for its use.

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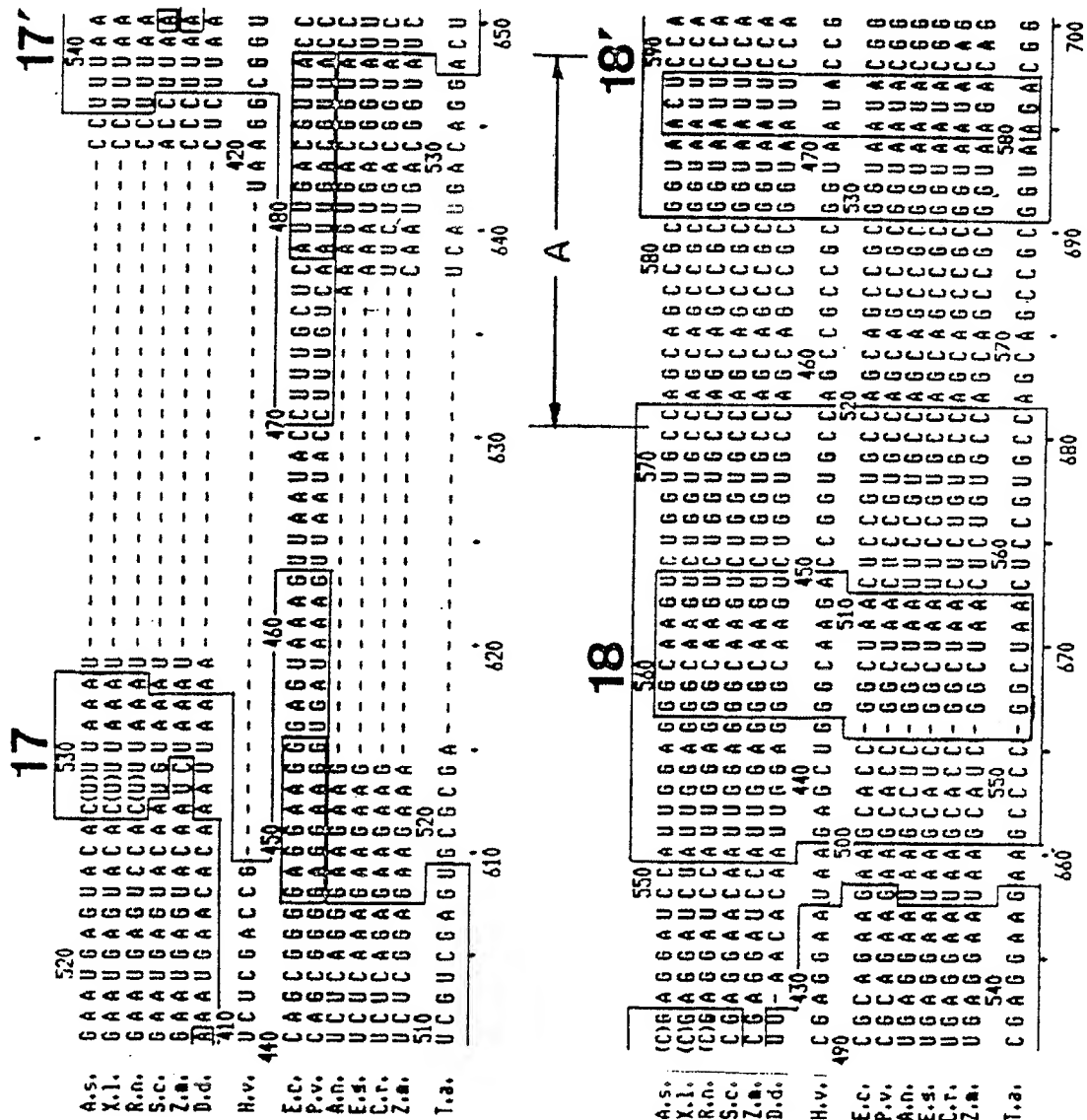
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FIG 1-1



	2'	E18-1	19
A.s.	600	610	630
X.1.	540	550	520
R.n.	500	510	580
S.c.	480	490	640
Z.m.	460	470	620
D.d.	440	450	600
H.v.	420	430	580
E.c.	400	410	560
P.v.	380	390	540
A.n.	360	370	520
E.s.	340	350	500
Z.m.	320	330	480
D.d.	300	310	460
H.v.	280	290	440
E.c.	260	270	420
P.v.	240	250	400
A.n.	220	230	380
E.s.	200	210	360
Z.m.	180	190	340
D.d.	160	170	320
H.v.	140	150	300
E.c.	120	130	280
P.v.	100	110	260
A.n.	80	90	240
E.s.	60	70	220
Z.m.	40	50	200
D.d.	20	30	180
H.v.	0	10	160
E.c.			140
P.v.			120
A.n.			100
E.s.			80
Z.m.			60
D.d.			40
H.v.			20
E.c.			0

FIG I-3

	E19-1	E19-1'
A.S.	700	730
X.I.	CC-AGCC	UUAACCGAGUGUCCU
R.N.	CC-AGCC	UUAACCGAGUGUCCU
S.C.	CC-AGCC	UUAACCGAGUGUCCU
Z.S.	CC-AGCC	UUAACCGAGUGUCCU
D.D.	CC-AGCC	UUAACCGAGUGUCCU
H.V.	CC-AGCC	UUAACCGAGUGUCCU
E.C.	CC-AGCC	UUAACCGAGUGUCCU
P.V.	CC-AGCC	UUAACCGAGUGUCCU
A.N.	CC-AGCC	UUAACCGAGUGUCCU
E.S.	CC-AGCC	UUAACCGAGUGUCCU
C.F.	CC-AGCC	UUAACCGAGUGUCCU
Z.M.	CC-AGCC	UUAACCGAGUGUCCU
T.S.	CC-AGCC	UUAACCGAGUGUCCU

	E19-1	E19-2	E19-2'
A.S.	740	760	770
X.I.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
R.N.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
S.C.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
Z.S.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
D.D.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
H.V.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
E.C.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
P.V.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
A.N.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
E.S.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
C.F.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
Z.M.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU
T.S.	6-6666	UUAACCGAGUGUCCU	UUAACCGAGUGUCCU

FIG I-4

E19-3									
A.S.	790	6	C	A	C	C	6	C	6
X.I.	800	-	C	6	A	6	C	C	6
R.n.	810	-	C	6	C	U	A	U	C
S.C.	820	-	C	6	C	U	A	U	C
Z.B.	830	-	C	6	C	U	A	U	C
D.d.	840	-	C	6	C	U	A	U	C
H.V.	850	-	C	6	C	U	A	U	C
E.C.	860	-	C	6	C	U	A	U	C
P.V.	870	-	C	6	C	U	A	U	C
A.n.	880	-	C	6	C	U	A	U	C
E.S.	890	-	C	6	C	U	A	U	C
C.F.	900	-	C	6	C	U	A	U	C
Z.B.	910	-	C	6	C	U	A	U	C
T.a.	920	-	C	6	C	U	A	U	C
P19-1									
	930	-	C	6	C	U	A	U	C
	940	-	C	6	C	U	A	U	C
	950	-	C	6	C	U	A	U	C

E19-3'									
A.S.	840	U	C	A	C	C	6	C	6
X.I.	850	U	C	A	C	C	6	C	6
R.n.	860	U	C	A	C	C	6	C	6
S.C.	870	U	C	A	C	C	6	C	6
Z.B.	880	U	C	A	C	C	6	C	6
D.d.	890	U	C	A	C	C	6	C	6
H.V.	900	U	C	A	C	C	6	C	6
E.C.	910	U	C	A	C	C	6	C	6
P.V.	920	U	C	A	C	C	6	C	6
A.n.	930	U	C	A	C	C	6	C	6
E.S.	940	U	C	A	C	C	6	C	6
C.F.	950	U	C	A	C	C	6	C	6
Z.B.	960	U	C	A	C	C	6	C	6
T.a.	970	U	C	A	C	C	6	C	6
P19-1'									
	980	-	C	6	C	U	A	U	C
	990	-	C	6	C	U	A	U	C
	1000	-	C	6	C	U	A	U	C

FIG I-5

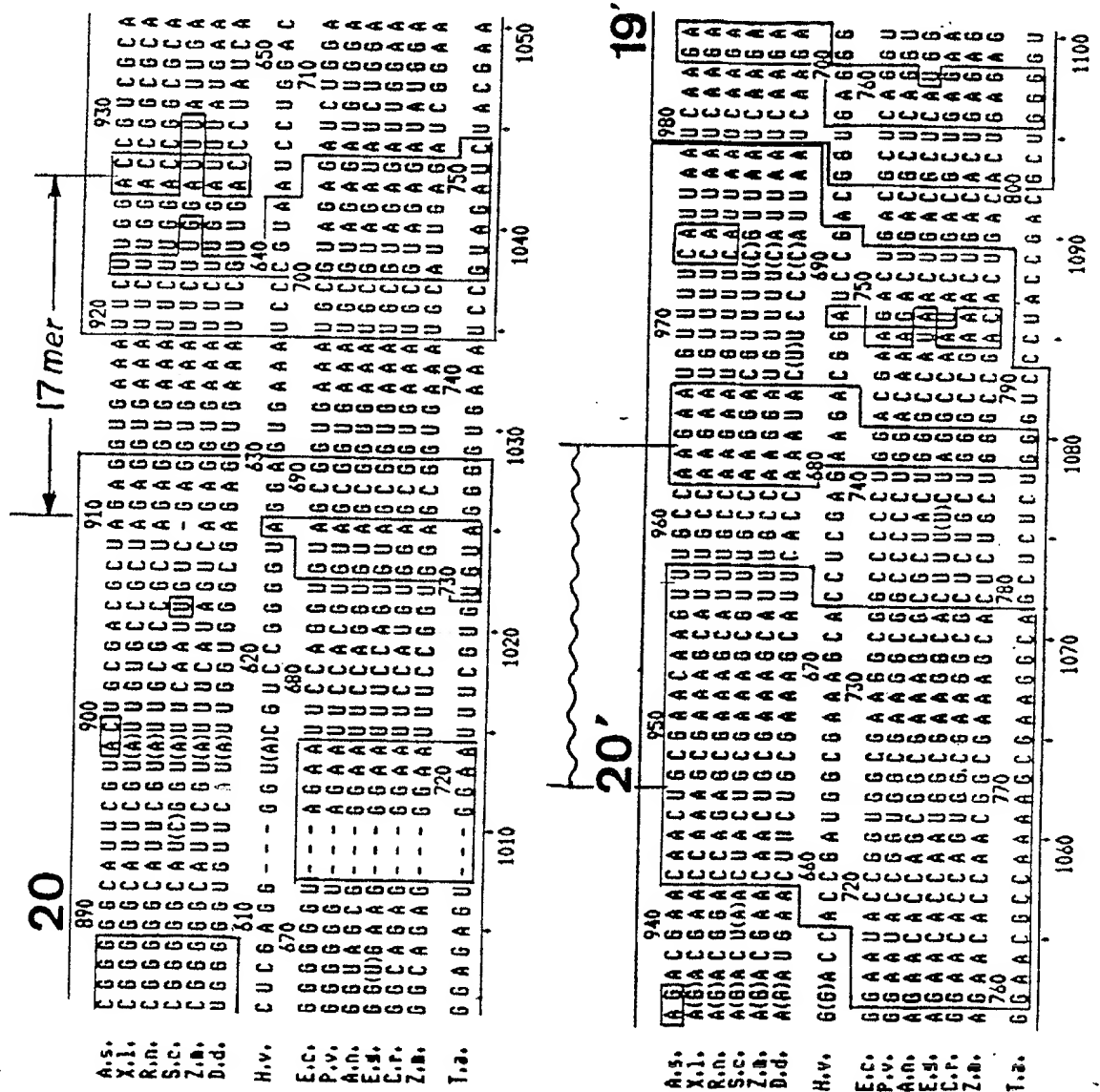
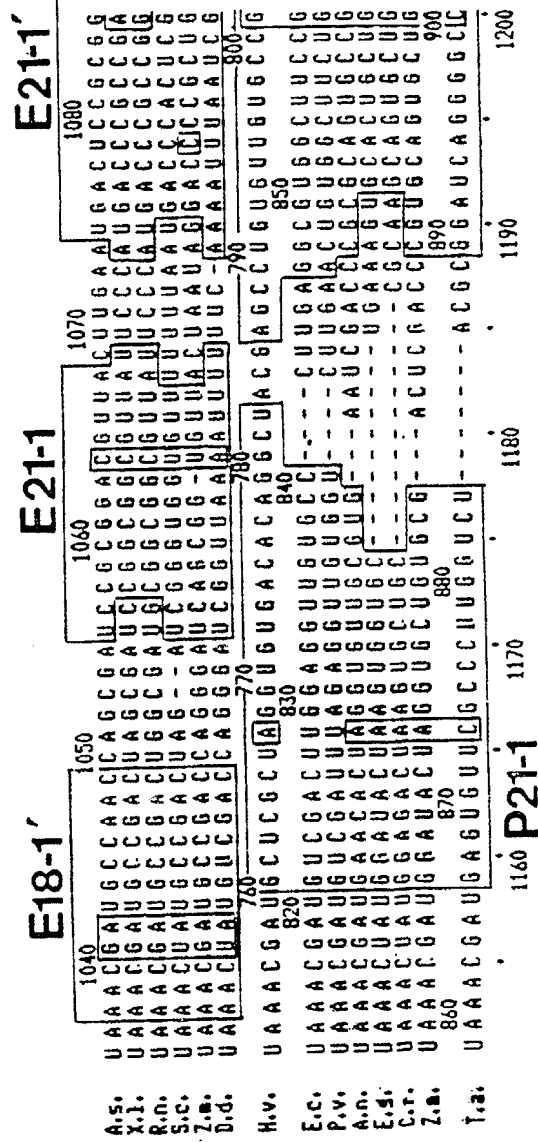
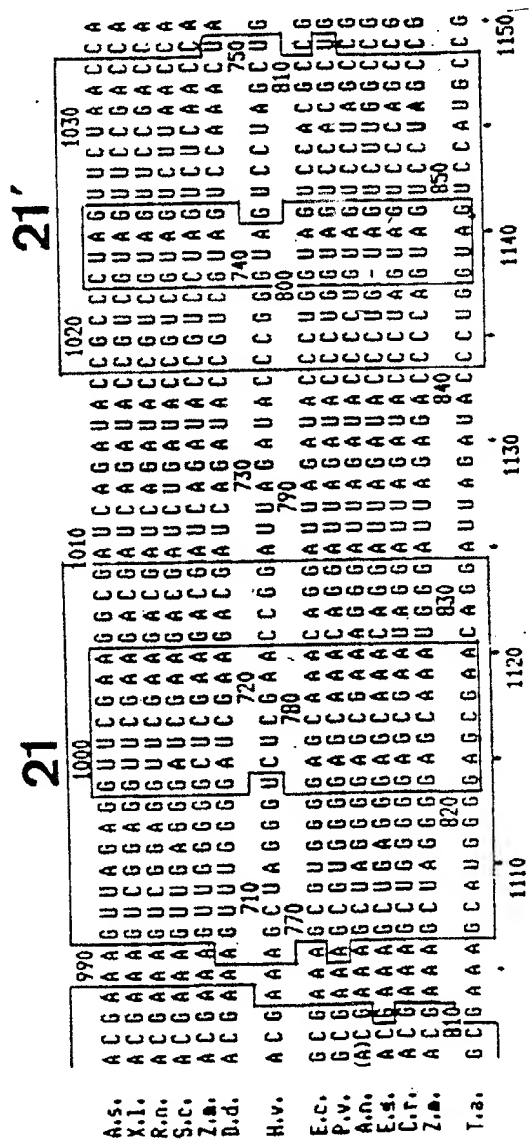




FIG 1-6






EUROPEAN PATENT APPLICATION


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
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
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 **Detecting Eukaryotic Microorganisms.**

 A cDNA probe, for use in detecting in a sample, under conditions of predetermined stringency, a target organism belonging to a strain of fungal microorganisms, and not detecting in the sample under such conditions a reference microorganism or any prokaryotic microorganism, can be constructed by:
determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from the target organism, the variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and having no corresponding region in prokaryotes; comparing the nucleotide base region in the variable region from the target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from the reference microorganism, and selecting as a useful probe site a subsequence within the determined sequence; and synthesizing a cDNA complementary to the useful probe site, the cDNA being the probe.

EP 0 335 633 A2

Description

DETECTING EUKARYOTIC MICROORGANISMS

This invention relates to detecting eukaryotic microorganisms.

Fungal infection, and particularly invasive systemic candidiasis, is a significant cause of morbidity and mortality in immunocompromised patients. It is important in treatment of invasive candidiasis to obtain a diagnosis early enough to permit application of effective antifungal therapy. Known approaches to premortem diagnosis of candidiasis include isolation of *Candida* species from blood cultures, serological tests for antibody to *Candida*, and direct detection of *Candida* antigens or *Candida* metabolites in serum.

L. de Repentigny et al. (1985), *J. Clin. Microbiol.*, vol. 21(6), pages 972-79, used enzyme immunoassay and gas-liquid chromatography to compare the concentrations of three metabolic markers for candidiasis in serum samples from normal blood donors and high risk patients with and without invasive candidiasis, and concluded that the best approach to diagnosis of invasive candidiasis entails obtaining blood cultures and carrying out serial assays for mannan in serum.

B.V. Kumar et al. (1985), *Infect. Immun.*, vol. 48(1), pages 806-12, showed that human antibodies in blood serum from patients with histoplasmosis reacted most frequently to antigens shared by three disparate fungi, *Histoplasma capsulatum*, *Candida albicans*, and *Saccharomyces cerevisiae*, while they reacted with low frequency to antigens specific for individual fungal species. Kumar et al. suggested that specific epitopes may be present as part of the shared antigens, and that monoclonal antibodies to, e.g., the mitochondrial fraction of *C. albicans* might be used for specific immunodiagnosis.

B.B. Magee et al. (1987), *J. Bacteriol.*, vol. 169(4), pages 1639-43, found differences in patterns of restriction fragment length polymorphisms ("RFLPs") for the ribosomal DNA ("rDNA") regions from clinical isolates of various species and strains of *Candida*, and suggested that RFLPs may be clinically useful in biotyping various strains of *C. albicans* or in distinguishing various species.

L. Nelles et al. (1984), *Nucleic Acids Research*, vol. 12(23), pages 8749-8768, proposed an alignment of fourteen small ribosomal subunit RNA ("srRNA") sequences of eukaryotic, archaeobacterial, eubacterial, chloroplastic, and plant mitochondrial origin, on the basis of the presence of conserved features in the sequences, such as regions of theoretical secondary structure formation. They identified a number of presumed double-stranded regions ("proposed helices"), and which were present in all the aligned sequences (that is, generally conserved "universal helices"), regions which occurred only in eukaryotic srRNAs (conserved among eukaryotes; "eukaryote-specific helices"), and other regions which were found only in prokaryotic srRNAs (conserved among prokaryotes; "prokaryote-specific helices"). Nelles et al. proposed a system for numbering the corresponding regions of the aligned sequences. For example, a presumed universal

helix, numbered 19 in the Nelles alignment, is at bases having nucleotide numbers 632 through 641 (A. salina-specific nucleotide numbering) in each of six eukaryotes in the alignment. They also identified "variable areas," in which the primary as well as the secondary structure appeared to be especially variable among the srRNAs, including, for example, "area V4" (bases 642-870).

Within V4 is a long eukaryotic region (bases 642-805) having low sequence conservation. This region is entirely absent from all the other aligned srRNA sequences from archaeobacterial, prokaryotic, eukaryotic mitochondrial, and eukaryotic chloroplast sources.

D.E. Kohne, Canadian Pat. No. 1 215 904, describes a probe which is complementary to rRNA from any bacteria but is not complementary to human cell rRNA, for detecting the presence of Mollicutes species in human cell culture and other types of bacterial cells; and a method for making the probe. Kohne uses whole *Mollicutes hominis* rRNA as a template for reverse transcriptase synthesis of radioactive cDNA; hybridizes the cDNA to *M. hominis* rRNA to ensure that the cDNA is indeed complementary to this rRNA; then the labeled cDNA is hybridized with an excess of human rRNA, and the fraction not hybridizing to human rRNA is recovered to provide the probe. Kohne also describes using this method, beginning with *Trypanosoma brucei* rRNA, to select a cDNA probe that is complementary to trypanosome rRNA but is not complementary to human rRNA, for detection and quantitation of trypanosomes in human samples by hybridization. Kohne also suggests comparing known nucleotide sequences of rRNA from different organisms to identify "group specific sequences similar to a specific group of organisms", and making as a probe a sequence complementary to such a sequence.

In general, the invention features, in one aspect, a method for constructing a cDNA probe for use in detecting in a sample under conditions of predetermined stringency a target organism belonging to a strain of fungal microorganisms and not detecting a reference microorganism and not detecting any prokaryotic microorganism. The method includes steps of: determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from the target organism; comparing the determined nucleotide base region in the variable region from the target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from the reference microorganism, and selecting as a useful probe site a subsequence within said determined sequence; and synthesizing a cDNA complementary to the useful probe site, the cDNA being the probe.

The term "eukaryotic microorganisms", as that term is used herein, includes, for example and without limitation, fungi, protozoa, and mammalian cells; the term does not include chlamydia, mycoplasma and the like organisms, such as archaeobac-

teria, intermediate between the conventional eukaryotes and prokaryotes. The term "fungal microorganisms", as that term is used herein, includes, for example and without limitation, molds and yeasts.

The term "variable region", as used herein, is a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and that has no corresponding region in prokaryotes. "Corresponding regions", as used herein, are regions of srRNA sequences which, when aligned as in the alignment proposed by Nelles et al., have the same positional relationships with respect to conserved regions, particularly with respect to proposed secondary structures.

In preferred embodiments, the variable region includes at least a part of the region immediately adjacent the 3' end of universal helix 19, that is, it includes at least a part of the region whose bases are assigned numbers 642 through 805 (*A. salina*-specific numbering) in the Nelles et al. alignment; the target organism is a strain of yeast, particularly a strain of yeast pathologic to humans, more particularly a strain of *Candida*, and still more particularly a strain of *Candida albicans*; and the probe has a nucleotide base sequence including at least about 15 consecutive nucleotides selected from the sequence

5' GGAAAGGCTCGGCTGGGTCCAGTACGCAT-
CAAAAAGATGGACC 3'.

The invention features, in another aspect, an iterative method for constructing a cDNA probe for use in detecting in a sample a target organism belonging to a strain of fungal microorganisms and not detecting any of a plurality of reference microorganisms and probe not detecting any prokaryotic microorganism. The iterative method includes steps of: constructing a cDNA as summarized above and testing it for hybridization with RNA from the reference organisms to determine whether it has the desired specificity; if so, then the cDNA is the probe; but if not, then a refined probe is made by determining the base sequence in the variable region of srRNA from a next one of the reference microorganisms; comparing it with the corresponding base sequences already determined and selecting as a refined useful probe site a subsequence within the determined sequence, and synthesizing a refined cDNA complementary to the refined useful probe site; and testing it for hybridization with RNA from the reference organisms to determine whether it has the desired specificity. If so, then this first refined cDNA is the probe; but if not, the steps are repeated using a next one of the reference microorganisms, until a probe of the desired specificity is obtained.

The method of the invention provides diagnostic probes having as broad a specificity as is desired for a particular use; probes for detecting particular strains or species of fungal microorganisms can be obtained, as well as probes useful for screening groups of strains or species of fungal microorganisms.

In another aspect, the invention features a nucleic acid probe that detects, under conditions of predetermined stringency, the presence in a sample of

a first predetermined strain of fungal microorganism and fails to detect under those conditions the presence in the sample both of a second predetermined strain of fungal microorganism and of any prokaryotic organism. The probe includes a nucleic acid sequence capable of hybridizing, under those conditions, with a subsequence of nucleotide bases including a portion of a nucleotide base sequence of said first predetermined strain, and failing to hybridize, under those conditions, with a second subsequence of nucleotide bases including a corresponding portion of a nucleotide base sequence of the second predetermined strain. The corresponding nucleotide base sequences from the two strains correspond to a variable region that is present in eukaryotic small subunit ribosomal RNA and is not present in prokaryotic small subunit ribosomal RNA.

In another aspect, the invention features a method for detecting in a sample an organism belonging to a strain of fungal microorganisms, the method including providing a probe of the invention, deriving RNA from the sample, contacting the probe with the RNA under conditions that allow the probe to hybridize with small subunit rRNA to form hybrid nucleic acid complexes, and detecting the hybrid nucleic acid complexes as an indication of the presence in the sample of the organism. In another aspect, the invention features a kit, including a probe of the invention, and instructions for its use.

The probes of the invention can be used to detect both viable and non-viable organisms in pure or mixed culture, or directly without culturing in complex samples such as clinical samples or food-related material. The diagnostic test using these probes can be highly sensitive, because the srRNA target molecules are present in high numbers in each cell.

Preferred embodiments of the invention will now be described with reference to the accompanying drawings, in which:

Fig. 1 is a portion of the sequence alignment of srRNAs from a variety of organisms, as proposed by L. Nelles et al. (1984), *Nucleic Acid Research*, vol. 12(23), pages 8749-68.

Fig. 2 is a *Candida albicans* srRNA cDNA sequence, as determined by direct RNA sequencing.

Fig. 2A is an RNA sequence deduced from a portion of the cDNA sequence in Fig. 2, aligned with a corresponding portion shown in Nelles et al. (1984) of the *Saccharomyces cerevisiae* srRNA sequence.

Fig. 2B is the sequence of a synthetic oligomeric cDNA probe complementary to a useful probe site of the RNA sequence shown between arrows in Fig. 2A.

The abbreviations used in the figures are as follows: A, adenine; T, thymine; G, guanine; C, cytosine; U, uracil; M, either cytosine or adenine; R, either guanine or adenine; K, either guanine or thymine; a, adenine (uncertain); t, thymine (uncertain); A.S., *Artemia salina*; X.L., *Xenopus laevis*; R.n., *Rattus norvegicus*; S.c., *Saccharomyces cerevisiae*; Z.m. [upper], *Zea mays*; D.d., *Dictyostelium discoideum*; H.V., *Halobacterium volcanii*; E.c., *Escheri-*

chia coli; P.v., *Proteus vulgaris*; A.n., *Anacystis nidulans*; E.g., *Euglena gracilis* chloroplast; Z.m. [lower], *Zea mays* chloroplast; T.a., *Triticum aestivum* mitochondrion. In figure 1, smaller numbers are nucleotide numbers specific for *A. salina*, *H. volcanii*, *E. coli*, and *T. aestivum* mitochondria srRNA, and are placed above those sequences. The small numbers at the bottom refer to a general numbering system for this alignment. All nucleotide numbers in this application refer only to the *A. salina*-specific numbering appearing at the top. Labeled boxes identify sequences proposed by Nelles et al. as representing regions of common potential secondary structure.

Method of Constructing the Probe

Generally, a probe for detecting a particular eukaryotic organism ("target" organism) is made according to the invention by determining the sequence of nucleotide bases in a region ("variable region") of the srRNA from the target organism that is poorly conserved among eukaryotes and that has no corresponding region in prokaryotes; comparing that determined sequence with the corresponding known sequence from a reference organism whose presence is not to be detected; identifying for use as a probe target sequence ("useful probe site") a subsequence within the determined sequence that is substantially different from the corresponding subsequence in the reference organism; and synthesizing for use as the probe a single-stranded cDNA complementary to the identified probe target sequence. A detailed protocol for carrying out the method follows. One skilled in the art will appreciate that variation in the particulars of the protocol can be made without departing from the invention.

1) Determination of an rRNA sequence of the target organism in a region that is poorly conserved among eukaryotes and that has no corresponding region in prokaryotes ("variable region").

With reference now to Fig. 1, showing a part of the alignment scheme proposed by Nelles et al. (1984), the nucleotide base sequences of the srRNAs from a variety of eukaryotic organisms have a highly variable region, encountered after (i.e., toward the 3' end of the srRNA, and having higher sequence alignment numbers in the Nelles et al. proposed alignment) universal helix 19, for which there is no corresponding region in prokaryotic organisms. The bases in this region are assigned alignment numbers 642 through 805 in the Nelles et al. scheme, and the region is termed herein the "642-805 region".

A modification of the sequencing technique described by Lane et al. (1985) *Proc. Natl. Acad. Sci. USA*, vol. 82, pages 6955-6959, is employed to determine the srRNA sequence of the particular eukaryotic organism to be detected (the "target organism") in the region between bases numbered 642 and 805 in the Nelles et al. scheme (the "642-805 region"). A 17-base DNA oligomer (5' TTGGCAATGCTTCGC 3'), which is complementary to a strongly (although not completely) conserved eukaryotic region, that is, bases numbered

947 through 963 in the Nelles et al. scheme, wavy line, Fig. 1 (the "947-963 region"), was used as a specific primer for sequencing the region of interest by reverse transcriptase catalyzed primer extension. When hybridized with a preparation of total cellular RNA from the target organism whose rRNA is to be sequenced, the 17-mer anneals to the site at the 947-963 region on the target organism 18S rRNA. The primer fails to anneal to any site on any contaminating prokaryotic (16S) rRNA under comparable stringency conditions. The total cellular RNA fraction may be prepared in any of several ways, but preferably it is prepared by subjecting cell homogenates from the organism to several phenol extractions and several chloroform extractions, followed by ethanol precipitation, NaCl precipitation, and dissolution in water or an aqueous buffer.

Then, cDNA is synthesized as described, for example, in Lane et al. *supra*, by primer extension catalyzed by AMV reverse transcriptase using mixtures of deoxyribonucleoside triphosphates (dNTP's) and di-deoxyribonucleoside triphosphates (ddNTP's). The cDNA sequence is read directly from gels showing the lengths of primer-extended products in which synthesis has been terminated by incorporation of a ddNTP precursor.

Primers other than the 947-963 region 17-mer also can be used; for example, the region including bases numbered 910 through 926 in the Nelles et al. scheme (the "910-926 region") is conserved among 5 of the 6 eukaryotes shown in Fig. 1 (5' GAGGUGAAUUCUUGGA 3'), while that of slime mold is the same except for a G at base 922 and a U at base 924. Thus, an equimolar mixture of complementary 17-mers containing two 2-fold redundancies at these positions (5' TC(C/A)A(A/C)GAATTT-CACCTC 3') can also be useful as a primer for primer extension synthesis of the 642-805 region.

Then the sequence of bases in the variable region from the organism to be detected, that is, the corresponding 642-805 region, is deduced from the cDNA sequence. It is preferred to deduce the rRNA sequence from the sequenced cDNA rather than from sequenced genomic subclones for several reasons: a) it is much faster since it involves only a small number of steps; b) it is much more efficient in that it minimizes the number of bases sequenced outside the region of interest; c) it is more relevant to development of diagnostic DNA probes in that the cDNA sequence obtained accurately reflects the sequence of the mature 18S rRNA molecules in cells, after all processing of larger precursor molecules has been completed. Although rDNA cistrons tend to be highly amplified in eukaryotic genomes (e.g. in *Saccharomyces cerevisiae* over 100 direct tandem repeats at a single genomic locus, all of which are transcriptionally active), the rRNA transcription products will be several orders of magnitude more highly amplified. Moreover, with simple disruption procedures used to release rRNA target molecules in a diagnostic test, the relative number of rDNA genes (complexed with chromosomal proteins) released will be very small or nonexistent. Thus, if a certain species were to contain an atypical rDNA gene structure associated with unusual splice

sites, for example, then deducing rRNA sequence from sequenced genomic clones might lead to erroneous predictions of the mature 18S rRNA sequence.

2) Comparison of the variable region sequence with the corresponding sequences of one or more organisms not to be detected.

The deduced rRNA sequence of the organism to be detected is now aligned with the corresponding rRNA sequence of the strain or species whose presence is not to be detected by the probe.

For example (this example is described in detail below), in order to design a probe specific detecting the yeast pathogen *Candida albicans* and not detecting *Saccharomyces cerevisiae* or any prokaryotes the 642-805 region sequence from *C. albicans* organism is first obtained as described above and then aligned with the corresponding sequence from *Saccharomyces cerevisiae*. The Example below provides a detailed description of how the 642-805 region sequence of *Candida albicans* can be determined using the general method described above.

3) Analysis of the aligned sequence(s) to determine a subregion containing a useful probe site ("UPS").

The choice of a UPS is made by inspecting the aligned sequences for mismatched bases. Generally the object is to select a UPS such that similarities in the sequence between the organism to be detected and the corresponding portions of the sequence in closely related species (the reference organism) are minimized. Probes designed according to this principle maximize the relative thermal instability, under predetermined conditions of stringency, of hybrids formed by hybridization with the cDNA probe of RNAs from the species not to be detected. One way to accomplish this, for example, is to choose the UPS so that predicted mismatch bases in hybrids are distributed uniformly along the sequence or are clustered near the middle rather than near the ends of the UPS sequence.

4) Synthesis and labelling of a single-stranded oligomeric cDNA probe complementary to the UPS region of the target organism.

The cDNA probe complementary to the target organisms UPS region is preferably synthesized using an automated nucleotide synthesizer. Labelling of the cDNA probe may be performed after synthesis on an automated nucleotide synthesizer by methods well known in the art. For example, the probe can be made to carry a non-radioactive label by using specially modified dNTP precursors during the nucleotide synthesis, as described generally in Rashtchian et al. (1987), Clinical Chemistry, vol. 33(a), pp. 1526-30; and in Applied Biosystems User Bulletin Number 38 (Nov. 3, 1986), pp. 1-5. Alternatively, unphosphorylated 5' ends can be labelled with ^{32}P following reaction with ^{32}P -gamma-ATP and polynucleotide kinase enzyme, according to conventional techniques as described generally in, for example, Maniatis et al. (1982), Molecular Cloning, A Laboratory Manual, Cold Spring Harbor,

New York.

Finally the probe is proven by testing it for specifically detecting in a sample the presence of the target organism and not detecting the organism whose presence is not to be detected.

Use

The cDNA probes of the invention are used to detect specific eukaryotic microorganisms, particularly *Candida* species of yeast and other fungi of importance to clinical testing or food testing, according to the following general steps:

1) Disruption of yeast cells or fungal hyphae.

The rRNA from organisms contained in the sample or in cultures derived from the sample is released from the cells by mechanical means, preferably by vigorous vortex mixing of the sample with acid-washed glass beads (about 0.45-0.50 mm diameter) as described generally in C.P. Kurtzman et al., (Review), In: J.F.T. Spencer et al., eds. (1983), Yeast Genetics: Fundamental and Applied Aspects, Springer-Verlag, New York, p. 140.

2) Denaturation of the rRNA.

The cellular homogenate is then treated in well-known fashion with denaturants or with heat (or both) to open up stem-loop or other secondary structures in the target rRNA.

3) Hybridization of sample with cDNA probe.

Then the sample is contacted with the cDNA probe under conditions of stringency in which the probe is known to hybridize with target rRNA. The denatured rRNA can be immobilized on nitrocellulose or nylon membranes and hybridized with the probe in solution. Alternatively, hybridization conditions in which both target and probe are in solution can be used.

4) Removal of non-hybridized probe.

For sample rRNA immobilized on membrane filters, non-hybridized probe can be removed by washing the filters in a solution of salt at a suitable concentration and at a suitable temperature according to well-known methods. For solution hybridizations, any method can be used, preferably a method employing an immobilized anti-DNA:RNA antibody, as described generally in Rashtchian et al., supra, to capture and immobilize RNA-DNA hybrid molecules while non-hybridized probe is removed by subsequent washes.

5) Detection of label in hybridized probe.

Probes labeled with radioactive isotopes can be detected in any of a number of ways, for example by some form of radioisotope counter or by exposure of photographic film. The signal from non-radioactive probes is detected using methods compatible with the type of labelling.

Many variations of the hybridization reaction are possible. For example, steps 1) and 2) can be combined by breaking cells directly in a buffer containing denaturants. For solution hybridizations, cells can be broken in a buffer that also contains the

labeled cDNA probe, followed by heat denaturation, then renaturation by cooling. In this case partial hydrolysis of the RNA with mild alkali or ribonuclease can enhance the accessibility of the probe to a site that might otherwise not be fully denatured.

Example

The following example demonstrates the manufacture of a *Candida*-specific rRNA probe according to the invention. Under selected stringency conditions the probe detects the presence of *Candida* species and does not detect other yeast species or other fungi or prokaryotes. The example is given by way of illustration and is not intended to limit the claims.

The probe was designed and made generally as described above, based on analysis of 18S rRNA sequence data from a strain of *Candida albicans*. The specificity of the probe was assayed against a variety of organisms, including other *Candida albicans* strains, *Candida* species, and less closely related yeasts and fungi. The organisms used in the specificity assay are listed in Table 1 ("Series A": *Candida* strains, *Listeria monocytogenes*, *E. coli*) and Table 2 ("Series B": other yeasts and fungi, human white blood cells).

TABLE 1
STRAINS (SERIES A)

ORGA- NISM	STRAIN	OTHER NAMES
1 <i>Candida albicans</i>	ATCC 18804*	
2 <i>Candida albicans</i>	ATCC 14053	
3 <i>Candida albicans</i>	ATCC 11006	<i>Candida stellatoidea</i>
4 <i>Candida glabrata</i>	ATCC 2001*	<i>Torulopsis glabrata</i> <i>Cryptococcus</i>
5 <i>Candida tropicalis</i>	ATCC 750*	
6 <i>Candida pseudo-tropicalis</i>	ATCC 4135*	<i>Candida kefyr</i> <i>Kluyveromyces fragilis</i> <i>marxianus</i> <i>Issatchenkia orientalis</i>
7 <i>Candida krusei</i>	ATCC 6258*	<i>Monilia parapsilosis</i>
8 <i>Candida parapsilosis</i>	ATCC 22019*	<i>Pichia guilliermondii</i> <i>Clavispora lusitanae</i>
9 <i>Candida guilliermondii</i>	ATCC 6260*	
10 <i>Candida lusitanae</i>	ATCC 42720	
11 <i>E. coli</i>	YMC	
12 <i>Listeria monocytogenes</i>	SLCC 6933, SLCC 6835	

* Type culture

TABLE 2
STRAINS (SERIES B)

	ORGA- NISM	STRAIN	OTHER NAMES
1	<u>Saccharo- myces cerevisiae</u>	X2180-1A	
2	<u>Schizo- myces pombe</u>	<u>ade6</u>	
3	<u>Schwan- niomyces castollii</u>	ATCC 26076	
4	<u>Yarrowia lipolytica</u>	ATCC 32338	<u>Candida lipolytica</u> <u>Saccharo- mycopsis lipolytica</u>
5	<u>Saccharo- myces uvarum</u>	comm. beer	<u>Saccharo- myces carlsber- gensis</u>
6	<u>Trichos- poron capitatum</u>	ATCC 10663	<u>Geotri- chum capitatum</u> <u>Blasto- schizo- myces capitatus</u>
7	<u>Trichos- poron bergelii</u>	ATCC 28592*	<u>Geotri- choides cutaneus</u> <u>Trichos- poron cutaneum</u>
8	<u>Trichophy- ton mentagro- phytes</u>	ATCC 28185	<u>Arth- roderma benha- mia</u>
9	<u>Trichophy- ton rubrum</u>	ATCC 28188	
10	<u>Alternaria alternata</u>	ATCC 13963	tomato black mold
11	Human White Blood Cells		

*Type culture

1) Preparation of *Candida albicans* total RNA.

Candida albicans (ATCC strain 18804) yeast cells exponentially growing at 30°C in 50 ml of liquid YEPD medium (1% yeast extract, 2% peptone, 2% dextrose) were distilled water-washed by low-speed centrifugation, suspended in 3 ml of an RNA extraction buffer (0.1 M LiCl, 0.1 M EDTA, 0.1 M

Tris-HCl, pH 7.4), and placed on ice. Two grams of acid-washed and distilled water-rinsed glass beads (0.45-0.50 mm diameter) were added and the mixture repeatedly shaken at the highest speed on a vortex mixer for 10 sec, and then placed in an ice bath for 10 sec, until a total time of 2 min had elapsed. Then, 0.05 ml of 10% sodium dodecylsul-
fate (SDS) and 1 ml of phenol (equilibrated with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added and the mixture agitated briefly. Following low-speed centrifugation to separate the phases, the top aqueous phase was removed and twice again extracted with phenol, then twice extracted with chloroform-isoamyl alcohol (24:1) and then adjusted to 0.3 M sodium acetate. Total nucleic acids (RNA and small plasmid DNA) were precipitated with 2 volumes of 100% ethanol. The precipitates were washed once with 70% ethanol and once with 100% ethanol, dried under vacuum, dissolved in sterile distilled water, and stored at -20°C. High molecular weight RNA was further separated from DNA and from low molecular weight RNA by precipitation with 2 M NaCl.

The resulting solution was diluted in distilled water and the UV absorption measured at 260 nm. Concentration of nucleic acid was determined on the basis of $A_{260} = 1.0$ (1-cm path length) for 50 micrograms per ml. Subsequent analysis by agarose gel electrophoresis revealed that greater than 99% of the nucleic acid stained by ethidium bromide is composed of a mixture of 18S and 25S subunit rRNAs.

2) Obtaining a *Candida albicans* srRNA cDNA sequence.

A cDNA sequence was obtained by generally following the primer-extension method described in Lane et al. (1985) *Proc. Natl. Acad. Sci. USA*, vol. 82, pages 6955-6959, using as a template the *Candida albicans* total RNA preparation described above and using as a primer the synthetic oligomeric DNA sequence 5' TTGGCAAATGCTTTCGC 3' (Fig. 1, wavy line). The cDNA sequence obtained is shown in Fig. 2.

3) Identification of a possible useful probe site ("UPS") and synthesis of a cDNA probe.

The deduced RNA sequence of the *C. albicans* srRNA in the 842-805 region then was compared with the corresponding region, shown in Nelles et al. (1984), from *Saccharomyces cerevisiae*, by aligning the sequences as shown in Fig. 2A.

One possible UPS, shown between arrows in Fig. 2A, was chosen by inspection of the aligned sequences. A 43-nucleotide oligomeric DNA complementary to this UPS (shown in Fig. 2B) was synthesized on an automated nucleotide synthesizer. The 43-mer was gel-purified and labeled at the 5' end with ^{32}P , following reaction with ^{32}P -gamma-ATP and T4 polynucleotide kinase enzyme. The end-labeled 43-mer *C. albicans* rRNA probe was gel-purified and assayed for specificity by hybridization reactions with various total RNA preparations from other eukaryotic organisms.

4) Assay for probe specificity.

The specificity of the 43-mer probe was tested empirically by first preparing total RNA from a collection of organisms (Tables 1 and 2) using methods the same as or similar to that described above for *C. albicans*. RNAs were denatured by dilution into a solution containing final concentrations of 7.5% formaldehyde and 10X SSC (1X SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), followed by heating to 50°C for 15 min., then quick cooling by immersion in an ice bath. Equal amounts (approx. 1 microgram) of denatured RNA from each strain were immobilized on 2 different nylon membrane filters. One filter was hybridized with the labeled 43-mer test probe, while the other was hybridized with a similarly end-labeled oligomeric DNA probe. This latter probe is an equimolar mixture of 4 different 18-mers that are complementary to the 572-589 region (region A, Fig. 1): 5' G(A/T)ATTACCGCGGC(G/T)GCTG 3'. This latter probe is used here as a control for the amount of denatured 18s rRNA immobilized on the filters because this region is very strongly conserved among small subunit rRNAs of all organisms (Lane et al. (1985) have described these 18-mers as "universal primers" for use in the cDNA primer-extension sequencing technique).

Filters were hybridized overnight (greater than 16 hours) under low-stringency conditions at room temperature in a simplified cocktail containing 1 mg/ml salmon sperm DNA (sonicated and heat-denatured), 0.5% SDS, 6X SSC, then subjected to low-stringency (6X SSC, 37°C) or high-stringency (0.1X SSC, 65°C) washes. The assay for hybridization was by exposure to X-ray film.

The 43-mer probe detected strains of only 3 other *Candida* species, besides the *Candida albicans* controls: *Candida tropicalis*, *Candida parapsilosis*, and *Candida guilliermondii* (weak positive). Following the initial low-stringency wash, all other strains in this collection (Tables 1 and 2) were essentially negative or only very slightly positive with the 43-mer probe. Following a high-stringency wash, the same pattern of hybridization obtained; the strains showing slight positives after low stringency show negative under high stringency. Results with the control 18-mers showed that all samples hybridized approximately equally well, showing that approximately equal amounts of hybridizable RNA were present on the filter.

Regarding the detection of *Candida* species and other unrelated yeasts and fungi comprising our test panel, the desirable negative test-probe results indicate a lack of hybridization rather than a lack of RNA, because in such cases the results of the positive control probe was positive. This indicates that hybridization took place and hence that hybridizable RNA was present. The sample derived from human white blood cells contains mostly DNA (only a small fraction is 18S and 28S rRNA). This is consistent with the observed results wherein the test probe did not hybridize but the positive control probe did, albeit weakly.

5) Assay for greater specificity under higher stringency.

Candida strains A1-A9 (Series A; Table 1) were examined by hybridization analysis with the 43-mer test probe under higher stringency conditions than those employed as described in section 4, above. For each strain, both a purified total RNA sample and a cell suspension sample were tested. The RNA sample was prepared as described above. Cells were suspended in distilled water and adjusted to a uniform turbidity corresponding to absorbance at 600 nm of 0.5. Equal aliquots of 0.3 ml of the cell suspension were adjusted to contain final concentrations of 10X SSC and 7.5% formaldehyde in a final volume of 1.0 ml, in 13mm x 100mm glass culture tubes. Two grams of glass beads were added and the tubes containing the mixture were placed in an ice bath, shaken at high speed on a vortex mixer for 30 seconds, incubated at 50°C for 15 min., and quickly cooled in an ice bath. 0.2 ml of the resulting homogenate (free of glass beads, which had quickly settled out) was immobilized on a nylon membrane filter and hybridized for 90 minutes with the labeled 43-mer *C. albicans* rRNA probe in 6X SSC at 65°C. The filters were then washed in 6X SSC at 65°C and assayed by exposure to X-ray film. The filters were also subsequently washed in 0.1X SSC at 65°C and assayed by exposure to X-ray film.

Under these conditions, *Candida tropicalis* was detected by the *C. albicans* rRNA-derived probe following the 6X SSC wash but not following the 0.1X SSC wash (approximately 99% of the radioactivity was lost in the high-stringency wash). Following the 0.1X SSC wash, *Candida parapsilosis* showed strong positive, while *Candida guilliermondii* showed weak positive. Moreover, positive signals were obtained from samples derived from whole yeast cells only in cases where the purified RNA samples are also strongly positive. *Candida* strains (Table 1) not detected with the 43-mer probe are negative for both purified RNA and whole-cell samples.

This *C. albicans*-derived srRNA 43-mer probe is thus demonstrably useful in a diagnostic assay for the presence of *Candida* yeast cells. The probe failed to detect non-*Candida* yeasts and other unrelated fungi or prokaryotic organisms (section 4 above), while at the same time detecting all tested *Candida* species known as actual or potential human pathogens, with the exceptions of *Candida glabrata*, *Candida krusei*, *Candida kefyr*, and *Candida lusitanae*. In addition, it detected *C. parapsilosis* and *C. guilliermondii*. Determination and alignment of sequences for *Candida parapsilosis* and *Candida guilliermondii*, using the general method described above, can provide both more specific probes and more general probes, including *Candida*-specific, yeast-specific, and fungi-specific probes.

Total RNA samples (5 micrograms each) from strains A1-A7 (*Candida* species), A11 (*E. coli*), B1 (*Saccharomyces cerevisiae*), B6 (*Trichosporon capitatum*), and B9 (*Trichophyton rubrum*) were denatured with formaldehyde and heat and then analyzed by electrophoresis through a 1.2% agarose gel containing formaldehyde. Ethidium bromide staining confirmed that all samples contained ap-

proximately equal abundances of major rRNA subunits (i.e. approximately uniform staining of 18S, 18S, 23S, and 25S bands). Following Northern blot transfer and immobilization on a nitrocellulose membrane the filter was probed with the *C. albicans*-derived 43-mer under the same hybridization and wash conditions as described above. Single bands of strongly positive hybridization were observed only in *Candida albicans* strains A1-A3 at a position corresponding to the migration of the ethidium bromide-stained 18S rRNA subunit. The 25S subunits exhibited no hybridization whatsoever, nor was there any hybridization detectable anywhere else throughout the fractionated total RNA samples. The 18S subunit from strain A5 (*Candida tropicalis*) exhibited a weaker hybridization signal than that from the other three strains. Fractionated total RNAs from all the other samples failed to exhibit any hybridization with the 43-mer probe. This example shows that positive hybridization exhibited by these total RNA samples was due to presence of 18S rRNA sequences homologous with and complementary to the *C. albicans*-derived 43-mer srRNA cDNA probe.

6) Further refinement of probe specificity.

The method of the invention can be used to make a more narrowly specific *Candida albicans* probe, that is, a probe that detects *C. albicans* and does not detect *C. parapsilosis* or *C. guilliermondii*, or to make a probe that detects a selected group of species, such as, for example, selected known actual or potential human pathogens, including *C. glabrata*, *C. krusei*, *C. kefyr*, and *C. lusitanae*.

Making a more narrowly specific probe requires elimination of "false positives". In the above example, detection of *C. parapsilosis* and *C. guilliermondii* can be eliminated by determining the variable region base sequence of the srRNA from one of the species, preferably *C. parapsilosis*, the more strongly positive one, aligning the base sequence with known variable region sequences, identifying a potential UPS by inspection of the alignments, and then synthesizing and testing a cDNA to the UPS. The potential UPS can be chosen as one which would hybridize to *C. albicans* but not to *C. parapsilosis* or to other species whose variable region sequences are known and included in the alignment. False positives, if any, detected in the test of this refined probe can be eliminated by iteration of the above steps. Only variable region sequences of particular false positives need be determined, and elimination of one such false positive can result in elimination as well of one or more others, so that not all sequences need be determined. A tremendous savings of steps and resources can thereby be realized.

Making a probe that detects a selected broader group of strains or species amounts to eliminating "false negatives". In the above example, detection of *C. glabrata*, *C. krusei*, *C. kefyr*, and *C. lusitanae* can be obtained by determining the variable region base sequence of one of these species, for example, *C. glabrata*, aligning the base sequence with known variable region sequences, identifying a known UPS

by inspection of the alignments, and then synthesizing and testing a cDNA to the UPS. The potential UPS can be chosen as one complementary to a DNA probe that would hybridize with both *C. albicans* and *C. glabrata*, but not with other species whose variable region sequences are known and included in the alignment.

False negatives, if any, detected in the test of this refined probe can be eliminated by iteration of the above steps. As is the case with refinement to eliminate false positives, elimination of one false negative can result in elimination as well of one or more others, so that here, too, not all sequences need be determined.

In this manner, a probe having a desired breadth of specificity for a particular species or strain or group of species or strains of fungal microorganisms can be made for any clinical or food-related setting.

Other Embodiments

Other embodiments are within the following claims. For example, probes can be made and used according to the invention for fungal microorganisms other than *Candida albicans*. Of particular interest are other fungal pathogens of humans, including, for example, yeast-like pathogens such as species of *Geotrichum*; non-yeast dermatophytes such as species of *Trichophyton*, *Microsporum*, and *Epidermophyton*; and systemic and non-systemic yeast pathogens such as species of *Cryptococcus*, *Coccidioides*, *Histoplasma*, *Blastomyces*, and other species of *Candida*.

Claims

1. A method for constructing a cDNA probe for use in detecting in a sample under conditions of predetermined stringency a target organism belonging to a strain of fungal microorganisms and not detecting in said sample under said conditions a reference microorganism, said probe not detecting in said sample under said conditions any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from said target organism, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and having no corresponding region in prokaryotes,

(2) comparing said nucleotide base region in said variable region from said target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said reference microorganism, and selecting as a useful probe site a subsequence within said determined sequence, and

(3) synthesizing a cDNA complementary to said useful probe site, said cDNA being

said probe.

2. A method for constructing a cDNA probe for use in detecting in a sample a target organism belonging to a strain of fungal microorganisms and not detecting any of a plurality of reference microorganisms, said probe not detecting any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from said target organism, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and said variable region having no corresponding region in prokaryotes,

(2) comparing said nucleotide base region in said variable region from said target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from one of said reference microorganisms, and selecting as a potential probe site a subsequence within said determined sequence,

(3) synthesizing a cDNA complementary to said useful probe site,

(4) contacting said cDNA with RNA from said reference microorganisms under conditions that allow said cDNA to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(5) determining whether hybrid nucleic acid complexes are formed during said contacting, and

(6) if hybrid nucleic acid complexes are not formed during said contacting, then said cDNA is said probe; but

(7) if hybrid nucleic acid complexes are formed during said contacting, then

(a) determining a nucleotide base sequence in said variable region of small subunit ribosomal RNA from a next one of said reference organisms,

(b) comparing said nucleotide base region in said variable region from said next reference organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said first reference microorganism and with the corresponding region of small subunit ribosomal RNA from said target microorganism and selecting as a potential next refined probe site a subsequence within said determined sequence,

(c) synthesizing a cDNA complementary to said next refined probe site,

(d) contacting said cDNA complementary to said next refined probe site with RNA from said reference microorganisms under conditions that allow said cDNA complementary to said next refined probe site to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(e) determining whether hybrid nucleic acid complexes are formed during said

contacting, and

(f) if hybrid nucleic acid complexes are not formed during said contacting, then said cDNA complementary to said next refined probe site is said probe; but

(g) if hybrid nucleic acid complexes are not formed during said contacting, then

(h) repeating steps (a) through (g).

3. A method for constructing a cDNA probe for use in detecting in a sample a target organism belonging to any of a plurality of predetermined strains of fungal microorganisms and not detecting any of a plurality of reference microorganisms, said probe not detecting any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from a first one of said predetermined strains of fungal microorganisms, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and said variable region having no corresponding region in prokaryotes,

(2) comparing said nucleotide base region in said variable region from said first strain with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from one of said reference microorganisms, and selecting as a potential probe site a subsequence within said determined sequence,

(3) synthesizing a cDNA complementary to said useful probe site,

(4) contacting said cDNA with RNA from said fungal microorganisms and with RNA from said reference microorganisms under conditions that allow said cDNA to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(5) determining whether hybrid nucleic acid complexes are formed with RNA from said predetermined strain of fungal microorganisms during said contacting and determining whether hybrid nucleic acid complexes are formed with RNA from said reference microorganisms during said contacting, and

(6) if hybrid nucleic acid complexes are formed with RNA from each of said predetermined strains during said contacting and are not formed with RNA from any of said reference microorganisms during said contacting, then said cDNA is said probe; but

(7) if hybrid nucleic acid complexes are not formed with RNA from any of said reference microorganisms and if hybrid nucleic acid complexes are not formed with RNA from one or more of said predetermined strains during said contacting, then

(a) determining a nucleotide base sequence in said variable region of small subunit ribosomal RNA from a next one of

said predetermined strains,

(b) comparing said nucleotide base region in said variable region from said next predetermined strain with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said one of said reference microorganisms, and selecting as a potential next refined probe site a subsequence within said determined sequence,

(c) synthesizing a cDNA complementary to said next refined probe site,

(d) contacting said cDNA complementary to said next refined probe site with RNA from said fungal microorganisms and with RNA from said reference microorganisms under conditions that allow said cDNA complementary to said next refined probe site to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

e) determining whether hybrid nucleic acid complexes are formed with RNA from said fungal microorganisms during said contacting and determining whether hybrid nucleic acid complexes are formed with RNA from said reference microorganisms during said contacting, and

(f) if hybrid nucleic acid complexes are formed with RNA from each of said predetermined strains and are not formed with RNA from any of said reference microorganisms during said contacting, then said cDNA is said probe; but

(g) if hybrid nucleic acid complexes are not formed with RNA from any of said reference microorganisms and if hybrid nucleic acid complexes are not formed with RNA from one or more of said predetermined strains during said contacting then

(h) repeating steps (a) through (g).

4. A method as claimed in claim 1, 2 or 3 wherein said variable region corresponds to the variable region adjacent the 3' end of universal helix 19.

5. A method as claimed in claim 1, 2 or 3 wherein said variable region corresponds to the 642-805 region.

6. A nucleic acid probe that detects, under conditions of predetermined stringency, the presence in a sample of a first predetermined strain of fungal microorganism and fails to detect, under said conditions, the presence in said sample both of a second predetermined strain of fungal microorganism and of any prokaryotic organism, said probe comprising a nucleic-acid sequence capable of hybridizing, under said conditions, with a subsequence of nucleotide bases comprising a portion of a nucleotide base sequence of said first predetermined strain, said nucleic acid sequence failing to hybridize,

under said conditions, with a second subsequence of nucleotide bases comprising a corresponding portion of a nucleotide base sequence of said second predetermined strain, said nucleotide base sequence of said first predetermined strain and said corresponding nucleotide base sequence from said second predetermined strain each corresponding to a variable region that is present in eukaryotic small subunit ribosomal RNA and is not present in prokaryotic small subunit ribosomal RNA.

7. A probe as claimed in claim 6, wherein said first predetermined strain is a strain of yeast, for example which is pathogenic to humans.

8. A probe as claimed in claim 6, wherein said first predetermined strain is a strain of *Candida*, preferably *Candida albicans*.

9. A probe as claimed in claim 6 wherein said first predetermined strain is *Candida albicans* ATCC strain 18804, and said second predetermined strain is a strain of *Saccharomyces cerevisiae*.

10. A probe as claimed in any one of claims 6 to 9, wherein said variable region is immediately adjacent the 3' end of universal helix 19.

11. A probe as claimed in any one of claims 6 to 9, wherein said variable region is the 642-805 region.

12. A nucleic acid probe capable of detecting in a sample, under predetermined stringency conditions, *Candida albicans*, said probe comprising a nucleotide base sequence of at least about 15 consecutive nucleotides selected from the sequence

5' GGAAAGGCTCGGCTGGGTCCAGTACG-CATCAAAAAGATGGACC 3'

13. A method for detecting in a sample an organism belonging to a strain of eukaryotic microorganisms, comprising providing a probe as claimed in any one of claims 6 to 12,

deriving RNA from said sample, contacting said probe with said RNA under conditions that allow said probe to hybridize with small subunit rRNA to form hybrid nucleic acid complexes, and detecting said hybrid nucleic acid complexes as an indication of the presence in said sample of said organism.

14. A kit, comprising a probe as claimed in any one of claims 6 to 12 and instructions for its use.

Claims for the following Contracting State: ES

1. A method for constructing a cDNA probe for use in detecting in a sample under conditions of predetermined stringency a target organism belonging to a strain of fungal microorganisms and not detecting in said sample under said conditions a reference microorganism, said probe not detecting in said sample under said conditions any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from said target organism, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and having no corresponding region in prokaryotes, 5

(2) comparing said nucleotide base region in said variable region from said target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said reference microorganism, and selecting as a useful probe site a subsequence within said determined sequence, and 10

(3) synthesizing a cDNA complementary to said useful probe site, said cDNA being said probe. 15

2. A method for constructing a cDNA probe for use in detecting in a sample a target organism belonging to a strain of fungal microorganisms and not detecting any of a plurality of reference microorganisms, said probe not detecting any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from said target organism, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and said variable region having no corresponding region in prokaryotes, 20

(2) comparing said nucleotide base region in said variable region from said target organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from one of said reference microorganisms, and selecting as a potential probe site a subsequence within said determined sequence, 25

(3) synthesizing a cDNA complementary to said useful probe site, 30

(4) contacting said cDNA with RNA from said reference microorganisms under conditions that allow said cDNA to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes, 35

(5) determining whether hybrid nucleic acid complexes are formed during said contacting, and 40

(6) if hybrid nucleic acid complexes are not formed during said contacting, then said cDNA is said probe; but 45

(7) if hybrid nucleic acid complexes are formed during said contacting, then 50

(a) determining a nucleotide base sequence in said variable region of small subunit ribosomal RNA from a next one of said reference organisms, 55

(b) comparing said nucleotide base region in said variable region from said next reference organism with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said first reference microorganism and 60

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with the corresponding region of small subunit ribosomal RNA from said target microorganism and selecting as a potential next refined probe site a subsequence within said determined sequence,

(c) synthesizing a cDNA complementary to said next refined probe site,

(d) contacting said cDNA complementary to said next refined probe site with RNA from said reference microorganisms under conditions that allow said cDNA complementary to said next refined probe site to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(e) determining whether hybrid nucleic acid complexes are formed during said contacting, and

(f) if hybrid nucleic acid complexes are not formed during said contacting, then said cDNA complementary to said next refined probe site is said probe; but

(g) if hybrid nucleic acid complexes are not formed during said contacting, then

(h) repeating steps (a) through (g).

3. A method for constructing a cDNA probe for use in detecting in a sample a target organism belonging to any of a plurality of predetermined strains of fungal microorganisms and not detecting any of a plurality of reference microorganisms, said probe not detecting any prokaryotic microorganism, comprising

(1) determining a nucleotide base sequence in a variable region of small subunit ribosomal RNA from a first one of said predetermined strains of fungal microorganisms, said variable region being a region of the small subunit ribosomal RNA that is poorly conserved among eukaryotes and said variable region having no corresponding region in prokaryotes,

(2) comparing said nucleotide base region in said variable region from said first strain with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from one of said reference microorganisms, and selecting as a potential probe site a subsequence within said determined sequence,

(3) synthesizing a cDNA complementary to said useful probe site,

(4) contacting said cDNA with RNA from said fungal microorganisms and with RNA from said reference microorganisms under conditions that allow said cDNA to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(5) determining whether hybrid nucleic acid complexes are formed with RNA from said predetermined strain of fungal microorganisms during said contacting and determining whether hybrid nucleic acid complexes are formed with RNA from said reference microorganisms during said contacting, and

(6) if hybrid nucleic acid complexes are formed with RNA from each of said predetermined strains during said contacting and are not formed with RNA from any of said reference microorganisms during said contacting, then said cDNA is said probe; but

(7) if hybrid nucleic acid complexes are not formed with RNA from any of said reference microorganisms and if hybrid nucleic acid complexes are not formed with RNA from one or more of said predetermined strains during said contacting, then

(a) determining a nucleotide base sequence in said variable region of small subunit ribosomal RNA from a next one of said predetermined strains,

(b) comparing said nucleotide base region in said variable region from said next predetermined strain with the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said first predetermined strain and the nucleotide base sequence in the corresponding region of small subunit ribosomal RNA from said one of said reference microorganisms, and selecting as a potential next refined probe site a subsequence within said determined sequence,

(c) synthesizing a cDNA complementary to said next refined probe site,

(d) contacting said cDNA complementary to said next refined probe site with RNA from said fungal microorganisms and with RNA from said reference microorganisms under conditions that allow said cDNA complementary to said next refined probe site to hybridize with small subunit ribosomal RNA to form hybrid nucleic acid complexes,

(e) determining whether hybrid nucleic acid complexes are formed with RNA from said fungal microorganisms during said contacting and determining whether hybrid nucleic acid complexes are formed with RNA from said reference microorganisms during said contacting, and

(f) if hybrid nucleic acid complexes are formed with RNA from each of said predetermined strains and are not formed with RNA from any of said reference microorganisms during said contacting, then said cDNA is said probe; but

(g) if hybrid nucleic acid complexes are not formed with RNA from any of said reference microorganisms and if hybrid nucleic acid complexes are not formed with RNA from one or more of said predetermined strains during said contacting, then

(h) repeating steps (a) through (g).

4. A method as claimed in claim 1, 2 or 3 wherein said variable region corresponds to the variable region adjacent the 3' end of universal helix 19.

5. A method as claimed in claim 1, 2 or 3 wherein said variable region corresponds to the 642-805 region.

6. A process for preparing nucleic acid probe that detects, under conditions of predetermined stringency, the presence in a sample of a first predetermined strain of fungal microorganism and fails to detect, under said conditions, the presence in said sample both of a second predetermined strain of fungal microorganism and of any prokaryotic organism, said probe comprising

a nucleic acid sequence capable of hybridizing, under said conditions, with a subsequence of nucleotide bases comprising a portion of a nucleotide base sequence of said first predetermined strain,

said nucleic acid sequence failing to hybridize, under said conditions, with a second subsequence of nucleotide bases comprising a corresponding portion of a nucleotide base sequence of said second predetermined strain, said nucleotide base sequence of said first predetermined strain and said corresponding nucleotide base sequence from said second predetermined strain each corresponding to a variable region that is present in eukaryotic small subunit ribosomal RNA and is not present in prokaryotic small subunit ribosomal RNA, the process comprising coupling successive nucleotides together and/or ligating oligo- and/or poly- nucleotides.

7. A process as claimed in claim 6, wherein said first predetermined strain is a strain of yeast, for example which is pathogenic to humans.

8. A process as claimed in claim 6, wherein said first predetermined strain is a strain of *Candida*, preferably *Candida albicans*.

9. A process as claimed in claim 6 wherein said first predetermined strain is *Candida albicans* ATCC strain 18804, and said second predetermined strain is a strain of *Saccharomyces cerevisiae*.

10. A process as claimed in any one of claims 6 to 9, wherein said variable region is immediately adjacent the 3' end of universal helix 19.

11. A process as claimed in any one of claims 6 to 9, wherein said variable region is the 642-805 region.

12. A process for the preparation of a nucleic acid probe capable of detecting in a sample, under predetermined stringency conditions, *Candida albicans*, said probe comprising a nucleotide base sequence of at least about 15 consecutive nucleotides selected from the sequence

5' GGAAAGGCTCGGCTGGGTCCAGTACG-CATCAAAAAGATGGACC 3', the process comprising coupling successive nucleotides together and/or ligating oligo-and/or poly-nucleotides.

13. A method for detecting in a sample an organism belonging to a strain of eukaryotic microorganisms, comprising

providing a probe preparable by a process as claimed in any one of claims 6 to 12, deriving RNA from said sample, contacting said probe with said RNA under conditions that allow said probe to hybridize with small subunit rRNA to form hybrid nucleic acid complexes, and

detecting said hybrid nucleic acid complexes as an indication of the presence in said sample of said organism.

14. A kit, comprising a probe preparable by a process as claimed in any one of claims 6 to 12 and instructions for its use.

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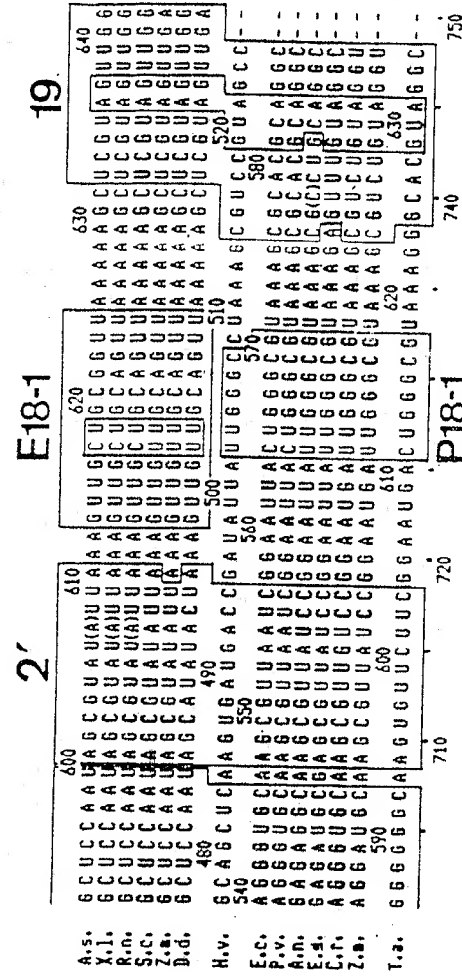
60

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FIG 1-1

FIG 1-2



A.s.	650	660	670	680	690
X.l.	U A U G G G U C	U C G G U C G G U C	U C G G U C G G U C	U C G G U C G G U C	U C G G U C G G U C
R.n.	A U C U U U G G G A	U C G A G C U G G C	U C G A G C U G G C	U C G A G C U G G C	U C G A G C U G G C
S.c.	A C U U U U G G G C	U C G G U C G G G C	U C G G U C G G G C	U C G G U C G G G C	U C G G U C G G G C
Z.a.	A C U U U U G G G C	U C G G U C G G G C	U C G G U C G G G C	U C G G U C G G G C	U C G G U C G G G C
D.d.	A G U U U A A G G G	U U A C C G G G U U	U U A C C G G G U U	U U A C C G G G U U	U U A C C G G G U U
H.v.	---	---	---	---	---
E.c.	---	---	---	---	---
P.v.	---	---	---	---	---
A.n.	---	---	---	---	---
E.a.	---	---	---	---	---
C.r.	---	---	---	---	---
Z.b.	---	---	---	---	---
T.a.	---	---	---	---	---
	760	770	780	790	800

FIG 1-4

E19-3		P19-1	
A.S.	790	910	950
X.I.	800		
R.O.	810		
S.C.	820		
Z.B.	830		
D.D.	840		
H.V.	850		
E.C.	860		
P.V.	870		
A.O.	880		
E.S.	890		
C.F.	900		
Z.B.	910		
T.A.	920		
E19-3'		P19-1'	
A.S.	840	960	1000
X.I.	850		
R.O.	860		
S.C.	870		
Z.B.	880		
D.D.	890		
H.V.	900		
E.C.	910		
P.V.	920		
A.O.	930		
E.S.	940		
C.F.	950		
Z.B.	960		
T.A.	970		

FIG I-5

